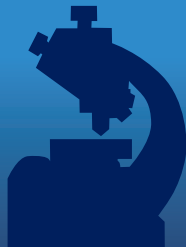


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# In this issue

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Clare Ward and Katharina Blattner report on the introduction of Point of Care Testing (POCT) which they presented at the 2010 NZIMLS Annual Scientific Meeting in the Bay of Islands. Prior to the introduction of POCT, specimens for analysis had to be sent to Whangarei but meant that there was no outside laboratory cover between Friday morning and Monday afternoon. This made it difficult to monitor patients, especially in the situation where it was important to know their electrolyte status. Introduction of POCT in Hokianga Hospital allows clinicians there to perform a small number of laboratory tests at the point of care. The proposed uses for POCT were for emergencies to guide acute treatment and in acute patients with the potential to become unwell. A critical element of the success of the implementation of the POCT system was the training and certification of staff and the development of ongoing quality assurance systems. This was well supported by the NDHB laboratory staff with getting the QA systems up and running ensuring standards are met and that training is ongoing. The introduction of POCT as an adjunct to more formal laboratory testing has meant that patients who live in Hokianga are far less disadvantaged because of their distance from these services. Clinicians caring for these patients can feel more confident in their decision making and are able to provide sound medical care to patients in urban areas.

Extended-spectrum  $\beta$ -lactamases (ESBLs) that mediate resistance to oxyimino-cephalosporins, such as cefotaxime, ceftazidime and aztreonam, have been observed worldwide. In this issue Mohd. Shahid provides a comprehensive review on CTX-M and AmpC  $\beta$ -lactamases that are currently of major concern, with particular reference to the situation in India. He also discusses about the development of novel cephalosporins, carbapenems and  $\beta$ -lactamase-inhibitors targeted against these resistance mechanisms. Mohd. Shahid submitted this review and with his peer-reviewed articles on the topic was awarded Fellowship of the NZIMLS.

Angela Horridge and colleagues evaluated three laboratory methods for the diagnosis of toxigenic *Clostridium difficile* infection, namely enzyme immunoassays (EIA) for glutamate dehydrogenase (GDH) and toxins A and B, real time PCR (*tcdC* gene) and stool culture. The *C. difficile* toxin A/B EIA kits failed to detect 3 of 15 true positive samples while the GDH EIA was not sensitive enough to be used as a screening assay in combination with any toxin A/B EIA. Combining the GDH EIA with any other test would increase workload, cost and turnaround time with no improvement in sensitivity. The best performing individual test in regards to sensitivity and turnaround time was the LightMix *C. difficile tcdC* RT-PCR. The best performing combination of assays was both a toxin A/B EIA on stool and culture. This would allow the reporting of 97.7% of results within 48 hours with 100% sensitivity and specificity.

Roberto Mazzaschi and colleagues present a case study of a non-mosaic ring chromosome 9 in a newborn baby. Routine karyotype analysis of cultured peripheral lymphocytes showed the presence of a ring chromosome 9 in 94% of cells while karyotype analysis of the parents confirmed that this chromosome rearrangement was *de novo*. A more detailed molecular karyotype analysis showed a loss of 6.7Mb and 1.3Mb from the short and long arms of chromosome 9. They conclude that it is likely that both the heart defect and the ambiguous genitalia in the baby were caused by the loss of genes on the abnormal ring chromosome.

In a letter to the Editor Stewart Clark raises a number of scientific concerns he has with a recent published article on the prevalence of extended spectrum  $\beta$ -lactamase among Gram-negative bacteria in the Journal. As per Journal policy, the author of that article was

given the opportunity to respond, which he did. Stewart Clark also felt the article was not of the standard he expects of the Journal. In an accompanying note the Editor points out that this article underwent peer review by two microbiology specialists and the Journal's Statistical Editor who commented that the statistical analysis was appropriate for the study. After revision, addressing the referees comments, it was deemed to be of sufficient scientific merit to be published in the Journal.

## Advertisers in this issue

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# Point of care testing in Hokianga Hospital or 'How to get a good night's sleep in a lab-free zone.'

*Katharina Blattner and Clare Ward*

## Introduction

The Hokianga Health Service is a rural health service which incorporates a rural hospital with acute bed capacity as a part of the services it offers. The hospital does not have its own laboratory which traditionally meant that diagnosis and management was heavily reliant on clinical acumen particularly in the out of hours settings, including weekends. In 2008 Point of Care testing was introduced to our service with the object of improving patient management. This addition to our service has been analysed and has shown to be beneficial both in terms of patient care and also in terms of clinician confidence and peace of mind.

## Rationale

Hokianga is an area on the West Coast of Northern New Zealand. It is regarded as a place of beginnings for the Tangata Whenua as it was here that Kupe, the great Polynesian navigator, arrived along with two taniwha, Arai Te Uru and Niniwa, who remain as guardians of the south and north sides of the Hokianga Harbour to this day. Hokianga (Mangungu) was also the place where the second signing of the Treaty of Waitangi took place on February 12, 1840. Hokianga is historically and culturally rich but today is economically poor with a deprivation index of which has similar implications as for other economically high needs communities in terms of access to resources including education, employment and health services.



The Hokianga Health Service is a rural health service which is administered by a community trust, the Hokianga Health Enterprise Trust (HHET). It comprises a hospital and ten health clinics as well as community development, community health worker, dental and mental health services. HHET is the sole provider of health care services for the Hokianga. Our population of approximately 6500 people is around 75% Maori which means that we encounter a particular group of medical issues biased towards diabetes, gout, hypertension and early onset ischaemic heart disease. We also have a disproportionate number of people with valvular heart disease which is probably largely on the basis of rheumatic fever which was a significant problem in the past and continues to require vigilance in terms of detection and ongoing management. In addition cancer, in particular lung cancer, forms a large part of the

spectrum of disease managed within our service.

The HHET was formed in 1992 and its principles were to uphold the principles laid down by nurses and doctors of earlier times. Core to these is to provide health care which is free at the point of need. This was first set in place through the establishment of Hokianga as a Special Medical Area in 1941 under the leadership of Dr George McCall Smith. Later Special Medical Areas were also formed on the West Coast of the South Island, on the East Coast of the North Island, at Whangaroa to the East of Hokianga and also on the Chatham Islands and a few other places. All of these areas were places with sparsely distributed populations where it would have been uneconomic for a doctor to set up in practice.

In 1992 restructuring of the health service threatened to destroy both Hokianga's Special Medical Area status and also threatened the continuation of the hospital services. The community protested the proposed changes and in the process it was able to show that in fact it cost less per capita to provide our service which is free at point of need. The Trust is administered by community trustees who are elected by their various communities and also by some co-opted members. Its cultural make up reflects the cultural demographic of Hokianga.

Our ten health clinics are situated in clinics to the north, south, east and west of the hospital, which is in Rawene. The clinics lie on either side of a harbour. Transport between north and south sides of the harbour relies on a vehicular ferry for access. The ferry operates between the hours of 7am and 8pm so that at night the north side communities are relatively cut off although we can and do get special ferries out. Some of the north side communities will use Kaitiaki Hospital after hours. The most distant community would be about one hour away if you did not miss the ferry and about two hours away if you did.



A doctor attends the distant clinics once or twice a week and in between times the areas are covered by a community health nurse. Hokianga Hospital has 10 acute beds, 10 long stay beds and 2 to 3 maternity beds. It also has an emergency room and a seven days a week health clinic.

As the only health provider for the Hokianga, care is provided across the primary secondary interface with local doctors (general practitioners, rural hospital generalists) providing 24/7 medical services. Rawene Hospital has around 750 admissions per year of which around 20% are transferred, most to Whangarei Base hospital but some to Auckland (mainly cardiology cases). Thus Rawene hospital provides the bulk of acute care for its own community. In 2010 the hospital celebrated 100 years of existence on its current site.



From the point of view of laboratory diagnostic services one of the big issues has always been that of our relative spatial and temporal isolation. This has been an increasing problem as we have sought to provide first world care which relies on being able to diagnose and manage medical problems and to improve the health and long term outlook of our patients.

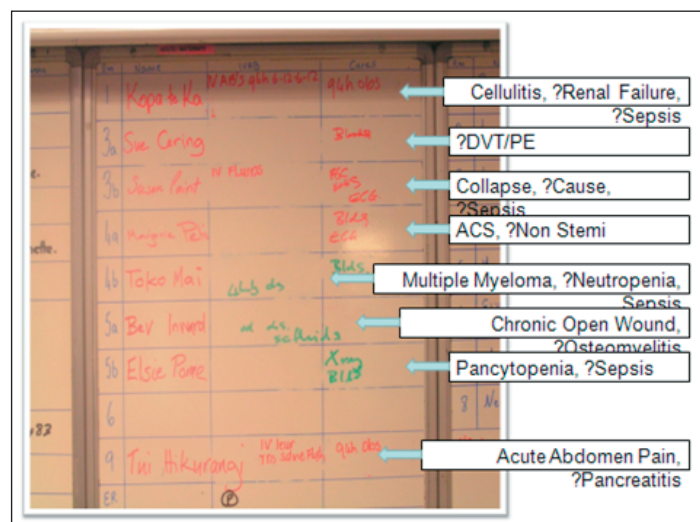
Up until about 1994 laboratory services were provided by Bay of Islands Hospital Laboratory situated at Kawakawa, which is about 80 km south of Rawene. A courier would take our samples down once daily on week day mornings and results would be sent back that evening. In 1994 we became computerized and at that time Bay of Islands Laboratory was unable to post electronic laboratory results so we therefore made the change to Northland Pathology Laboratory which again meant getting specimens down to Whangarei and getting at least some of the results back that evening. In practical terms one of our staff drives the specimens to Kaikohe every week day morning and from there a courier service takes them on to Whangarei.

The service is a good one but it is limited in that it operates only in office hours and we have no outside laboratory cover between 10am Friday morning and 4pm Monday afternoon. The turn-around-time (TAT) is thus up to 78 hours on a weekend. This could make it difficult to monitor patients, especially in the situation where it was important to know their electrolyte status. For this reason it has become imperative to find solutions that facilitate our ability to provide a safe and high quality service both in the clinics but more especially in our acute hospital setting.

Most standard guidelines for medical emergencies include basic lab tests as part of the initial assessment and management and to guide ongoing care. At the same time there is considerable variation around the country in terms of access to laboratory services for acute care (2). Some of this is obviously due to geography but some is due to healthcare structure/models of care, for example: most of the emergency care of urban patients is provided by specialists in Emergency Departments of larger hospitals where there is immediate access to lab tests. By contrast in most rural areas it is generalists/ GPs who provide the initial emergency care and they often have limited or no access to lab tests. Thus it is likely that regarding laboratory tests in acute illness, rural people not only

have significantly lower rates of intervention but also have a far greater waiting time from initial presentation until the lab test is done.

We know from our own situation in Rawene, that doctors in small rural hospitals in NZ are often faced with complex medical problems without immediate or even intermediate access to basic lab tests to help guide management including decisions around transfer of the patient. Many of the patients we see self-present at the front door of the hospital acutely unwell, with multiple co morbidities, on multiple medications often with an undifferentiated clinical picture. It is here that the rapid lab results are most helpful: in giving additional information to that gathered clinically e.g.: Has their renal function deteriorated? Are they anaemic? Are they over-anticoagulated? Are the electrolytes ok?



In 2008 a POCT analyser was installed at Rawene Hospital which allowed clinicians to perform a small number of lab tests at the point of care. (TAT 3-10 minutes.) The proposed uses for POCT were: i) Emergencies: to guide acute treatment, and ii) in acute patients with the potential to become unwell. The tests (Abbott Laboratories) included a chemistry panel (CHEM8: Na, K, Cl, iCa, Glu, BUN/urea, TCO<sub>2</sub>, Crea, Hct, AnGap, Hb), a blood gas, (CG8: pH, PCO<sub>2</sub>, PO<sub>2</sub>, Na, K, iCa, Gluc, Hct, HCO<sub>3</sub>, TCO<sub>2</sub>, BE, sO<sub>2</sub>, Hb), troponin I, INR and BNP.

At the same time we undertook a research project to look at the impact on our service on clinicians and the community. This project was funded by a Rural Innovation Fund grant and was a collaboration between Hokianga Health and the University of Otago. The project involved two studies, both of which have been published since elsewhere (3, 4).

The first involved analysis of quantitative data collected to measure change in clinical decisions associated with POCT use. POCT significantly improved diagnostic certainty with POCT results narrowing the differential diagnosis in 94% of patients. POCT resulted in a substantial change in treatment in 75% of cases. There was a marked impact on patient disposition, with a significant reduction in the number of transfers to base hospital and an increase in the number of discharges home. Admissions to Rawene Hospital remained relatively unchanged. POCT led to a change in disposition in 43% of patients. POCT also led to improved co-ordination of care across the primary-secondary and -tertiary interface with more appropriate as well as expedited transfers. For example, a patient with an acute renal problem was able to be transferred directly to the renal unit rather than going via the busy emergency department at Base hospital. POCT has enabled clinicians to better manage emergency cases and follow clinical guidelines whilst awaiting transfer to base hospital eg: in the case of diabetic ketoacidosis.

Another example of where POCT has helped clinicians to work to guidelines is in early recognition and management of Acute Coronary Syndromes/NONSTEMIs. The latest ACC/AHA guidelines for the management of patient with unstable angina/Non ST elevation myocardial infarction (5) ACC 2007(50)1-157) recommend a troponin result be available within 60 minutes of the test being performed. Thus these patients can be identified earlier, treated more appropriately and transferred for more timely intervention to CCU at Base hospital or if indicated directly to our interventional cardiology centre in Auckland .

The qualitative study examined in-depth understanding of the effects of the introduction of POCT on clinicians and the community and showed POCT had resulted in improved confidence and diagnostic self sufficiency of clinicians. It also revealed challenges. Carrying out the POCT tests and the QA requirements were time consuming. Having POCT has required higher standards of practice, with clinical staff having to up skill in order to do- but also interpret the test results. This increases the workload on an already stretched group of staff, especially after hours when staff numbers are limited. The study confirmed that POCT was used significantly more frequently during after-hours.ie: evenings and weekends when the TAT for regular lab tests is up to 78 hours. It is unlikely that having these small number of POC tests available has made any difference to our number of normal laboratory test requests: this is a very good service for the bulk of our work.

Obviously there are also many blood tests that are only available via the Whangarei laboratory and thus the TAT for these tests in the acute situation remains far too long. Our wish list for additional POCT includes: full blood count, CRP, liver function tests and amylase/lipase in particular. These few additional tests would, in our experience, contribute further to clinical decision making in the acutely unwell patient.

A critical element of the success of the implementation of our POCT system at Rawene (as for any POCT system) is the training and certification of staff and the development of ongoing quality assurance systems. We looked at the Quality Assurance, (QA) aspect as part of the POCT project and we have been very well supported by the NDHB laboratory staff with getting the QA systems up and running. It is the continued commitment of our nursing leaders, working together with NDHB lab staff that ensures standards are met and that training is ongoing.

The use of POCT in the rural hospital goes a long way in addressing the inequity in acute health care provision in rural areas allowing rural clinicians access to necessary and critical investigations in a clinically relevant TAT. This project has strengthened our resolve to continue working for equitable access to health services for our community.

Regarding transferability of our project, several factors that would need to be taken into consideration for a rural health service wanting to implement POCT. The travel time from Rawene Hospital to Base hospital and its laboratory service are key factors in the benefits associated with POCT at Rawene, as are the demographics of the population it serves. Other important factors are the relationship of the acute rural health service with its DHB and infrastructural differences.

In conclusion then, Hauora Hokianga serves a remote rural population which is distant from formal laboratory services. Because the nature of medicine is constantly changing and more and more can be provided to help with potentially life and health threatening conditions, we have become increasingly reliant on good laboratory diagnostic services. The introduction of POCT as an adjunct to more formal laboratory testing provided in laboratories in Kaitaia and Whangarei has meant that patients who live in Hokianga are far less disadvantaged because of their distance from these services. It has meant that clinicians and others caring for these patients can feel more confident in their decision making. In addition, they are able to provide sound medical care to a standard

which patients in urban areas have come to expect.



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## Author information

Clare Ward, MB ChB FRNZCGP FDRHMNZ, Medical Director<sup>1</sup>  
Katharina Blattner, MB ChB FRNZCGP FDRHMNZ, Rural Doctor<sup>1</sup>  
and Senior Clinical Lecturer<sup>2</sup>

<sup>1</sup>Hokianga Health, Private Bag 753, Kaikohe

<sup>2</sup>Department of General Practice and Rural Health, School of Medicine, University Otago, Dunedin

<sup>1</sup>Author for correspondence: [clare.ward@hokiangahealth.org.nz](mailto:clare.ward@hokiangahealth.org.nz)

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# $\beta$ -lactams vs. $\beta$ -lactamases - recent evolution of concurrent occurrence of multiple bla genes including *bla*<sub>CTX-M</sub> and *bla*<sub>ampC</sub>: an Indian scenario

Mohd. Shahid

## Abstract

Antibacterial agents have been used for treatment of infectious diseases since the 17<sup>th</sup> century. Amongst numerous defense mechanisms, the principal mechanism in bacteria is production of  $\beta$ -lactamase enzymes that hydrolyze the  $\beta$ -lactam ring resulting in production of biologically inactive product (1). To date, more than 500 different types of  $\beta$ -lactamases have been described on the Lahey-Clinic website (<http://www.laheyclinic.org/studies/webt.htm>), including the CTX-M and AmpC type  $\beta$ -lactamases. Carbapenems were reported to be active against strains that harbor these enzymes but recently several researchers have described the emergence of resistance due to a dual mechanism (2).

Extended-spectrum  $\beta$ -lactamases (ESBLs) that mediate resistance to oxyimino-cephalosporins (eg. cefotaxime, ceftazidime, and aztreonam) have now been observed worldwide (3). Amongst ESBLs, CTX-M  $\beta$ -lactamases are now some of the most common  $\beta$ -lactamases responsible for antibiotic resistance worldwide. To date, nearly 113 different variants of CTX-M  $\beta$ -lactamases and 107 variants of AmpC  $\beta$ -lactamases have been reported (<http://www.laheyclinic.org/studies/webt.htm>; date last accessed- 24<sup>th</sup> Jan 2011). An Indian study recently showed co-existence of *bla*<sub>CTX-M</sub> and *bla*<sub>ampC</sub> (4). Emergence of multiple *bla* genes in Enterobacteriaceae (5) and *Citrobacter* spp. has also been reported (6).

In response to the challenge of emerging resistance, a number of novel  $\beta$ -lactams (eg. ceftobiprole, ceftaroline) and  $\beta$ -lactamase-inhibitors have been developed, some of which are undergoing clinical trials (1). Other newer cephalosporins and carbapenems are still under developmental phase.

This article will provide a brief review on CTX-M and AmpC  $\beta$ -lactamases that are currently of major concern, with particular reference to the situation in India (6). Finally, there will be a brief discussion about development of novel cephalosporins, carbapenems, and  $\beta$ -lactamase-inhibitors targeted against these resistance mechanisms.

**Key words:** Extended-spectrum  $\beta$ -lactamases; carbapenems; *bla*<sub>CTX-M</sub>; *bla*<sub>ampC</sub>; AmpC; CTX-M; India.

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## ESBLs and their classification

ESBLs are enzymes that are able to hydrolyze oxyimino-cephalosporins (eg. cefotaxime, ceftazidime and aztreonam), but not cephamycins or carbapenems (3). Various classifications schemes for ESBLs are currently available, and of those the "Ambler classification" is the most widely used (7). According to this scheme,  $\beta$ -lactamases are classified into four classes (A, B, C and D) on the basis of their amino acid homology. Classes A, C, and D are serine  $\beta$ -lactamases, and the class B enzymes are metallo- $\beta$ -lactamases (MBLs). Classes A and C are the most frequently occurring ESBLs among bacteria (7).  $\beta$ -lactamase inhibitors (eg. clavulanic acid, sulbactam and tazobactam) inhibit Class A enzymes but do not inhibit class D enzymes.

The majority of ESBLs identified in clinical isolates were of SHV

or TEM types (3), and can further be subdivided on the basis of their activities against the two cephalosporins ceftazidime and cefotaxime. Ceftazidimases have higher levels of hydrolytic activity against ceftazidime than against cefotaxime while conversely cefotaximases have higher levels of hydrolytic activity against cefotaxime than against ceftazidime (8).

## CTX-M class of ESBLs

Oxyimino-cephalosporins, such as cefotaxime and ceftazidime, were introduced in early 1980s to treat infections caused by Gram-negative bacilli which were found resistant to established  $\beta$ -lactams and produced class A, C, and D type  $\beta$ -lactamases (1). The repetitive and increased use of these antibiotics led to appearance of resistant strains that overproduced class C enzymes (9) and/or ESBLs of both classes A and D (3,10). Class A ESBLs hydrolyze oxyimino-cephalosporins and aztreonam, but not 7- $\alpha$ -substituted  $\beta$ -lactams, and are generally susceptible to  $\beta$ -lactamase inhibitors such as clavulanate, sulbactam, and tazobactam (1).

Many groups of  $\beta$ -lactamases have hydrolytic profiles similar to those of the TEM and SHV mutants but have different evolutionary histories. These non-TEM and non-SHV plasmid-mediated class A ESBLs have been sub-divided into cefotaximases (CTX-M, SFO-1, and BES-1 type), and ceftazidimases (PER, VEB, TLA-1, and GES/IBC types) (11,12). According to recent reports, CTX-M  $\beta$ -lactamases are the most widespread ESBL enzymes worldwide (3,13) and also in the Indian subcontinent (5,6,14).

## Classification and origin of CTX-M $\beta$ -lactamases

Over 100 different variants of the CTX-M  $\beta$ -lactamases have been described (<http://www.laheyclinic.org/studies/webt.htm>), and these have been divided into five clusters based on their amino acid identities (12): the CTX-M-1 group includes CTX-M-1, -3, -10, -11, -12, -15 (UOE-1), -22, -23, -28, -30, -28, -29, -30, -32, -33, -36, -54, (15-20); the CTX-M-2 group includes CTX-M-2, -4, -5, -6, -7, -20, -31, -44 (previously Toho-1) and FEC-1 (21-27); the CTX-M-8 group includes CTX-M-8, -40 (28); the CTX-M-9 group includes CTX-M-9, -13, -14 (UOE-2 and Toho-3), -16, -17, -18, -19, -21, -24, -27, -45 (previously Toho-2), -46, -47, -48, -49, -50 (27,29-37); and the CTX-M-25 group includes CTX-M-25, -26, -39, -41 (18,20,34,36,38-40). CTX-M-14 was later reported identical to CTX-M-18, and CTX-M-55 to CTX-M-57, as per Lahey-clinic website (<http://www.laheyclinic.org/studies/webt.htm>).

A plasmid mediated  $\beta$ -lactamase able to hydrolyze extended-spectrum  $\beta$ -lactams was first discovered in Germany in 1983 (41). Class A ESBLs were initially reported in second half of the 1980s and their dissemination increased since 1995 (3,9,21). CTX-M was first discovered in Japan in 1986 and was designated FEC-1 (15). In 1989, a non-TEM, non-SHV ESBL obtained from an *E. coli* strain, from Germany, was for the first time designated as CTX-M-1 (42). Since then, nearly 113 CTX-M variants have been identified and designated from CTX-M-1 to CTX-M-113 (<http://www.laheyclinic.org/studies/webt.htm>). Figure 1 shows the clonal relationship of various CTX-M types whose accession numbers were available on Lahey-clinic website and sequences could be retrieved from GenBank at the time of writing this review article.





Figure 1. Neighbour joining tree self-drawn using the Multiple Sequence Alignment tool of CLUSTALW software (<http://align.genome.jp/sit-bin/clustalw>).

## International/national burden of CTX-M $\beta$ -lactamases

CTX-M-producing isolates are now endemic in most European countries, Asia and South America. Some CTX-M enzymes have a more restricted distribution (eg. CTX-M-1 in Italy (43), CTX-M-2 in South America, Japan and Israel (12,44), CTX-M-9 and CTX-M-14 in Spain and United Kingdom (45-47)). CTX-M-15 has been found worldwide (43,48-51). ESBLs have been known since 1982 but their types, prevalence and distribution in Europe have changed since 2003, following similar but earlier shifts in South America and Asia (52). Since the first description of CTX-M-15 in 2001 from India (18), it has spread rapidly worldwide (53-58). Recently, CTX-M-15-producing *K. pneumoniae* have also been reported from the southern part of India (59), as it has already been reported previously from northern India (14). In 2008,  $bla_{CTX-M-28}$  was reported for the first time from India (60). Till today, CTX-M-15 continues to be reported as the most prevalent CTX-M-type ESBL, especially in India, in Enterobacteriaceae (5) and *Citrobacter* spp. (6).

## Detection of CTX-M ESBLs by phenotypic methods

Ceftazidime resistance is used as a marker of ESBL production, as it is hydrolysed by both TEM and SHV type beta-lactamases. However, many CTX-M-producing strains do not show in vitro resistance to ceftazidime by standard methodologies. Therefore, susceptibility to cefotaxime should also be tested to look for CTX-M production (61,62). Alternatively, susceptibility to cefpodoxime (which is hydrolyzed by TEM, SHV, and CTX-M ESBLs) may be used as a screening test to detect most types of ESBL, although there is a significant rate of false-positive results.

In all cases, class A ESBL production should be confirmed by appropriate synergy tests (4). One of the most commonly used synergy tests is the "double disc synergy test (DDST)" originally described by Brun-Buisson *et al.* (63) and subsequently by Jarlier *et al.* (64). We have evaluated the *in vitro* efficacy of ceftriaxone/sulbactam (a combination that has recently been launched in India) in *E. coli* harbouring  $bla_{CTX-M-15}$ . We observed that 96.3% of these isolates were susceptible to ceftriaxone/sulbactam while only 12.9% were susceptible to ceftriaxone/tazobactam (65). This finding was in contrast to previous international (66) and national (67) studies which reported better activity of  $\beta$ -lactam / tazobactam combination against *E. coli* isolates. We also observed that piperacillin-tazobactam (or tazobactam alone) could be a better indicator when combined with ceftriaxone and cefotaxime for phenotypic detection of ESBLs, especially in CTX-M-producers (65).

## Detection of CTX-M ESBLs by molecular methods

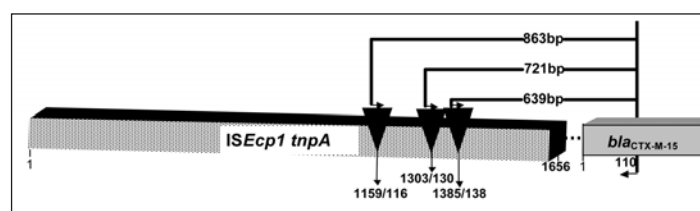
Multiplex PCR protocols for detection of  $bla_{CTX-M}$  genes are now widely used, with modifications adopted in individual laboratories (4,6,14,68). Subsequent to CTX-M-genogroup detection, precise CTX-M-genotypes could be identified either by using monoplex PCR for specific types, by reverse line hybridization, or by gene-sequence analyses (14,68). Molecular analysis of CTX-M genes can also be done by using different multiplex PCR-protocols (where different genogroups can be identified in a single reaction by using different sets of primers) as described by Woodford *et al.* (69) and by Xu *et al.* (70). Denaturing high performance liquid chromatography (dHPLC) is a recently introduced technique and can be used to detect genetic variation (70). Very recently, the combination of real time PCR with pyrosequencing has proved to be a powerful tool for epidemiological studies of CTX-M producers (71,72). We applied novel reverse line hybridization assay for the first time in Indian Enterobacteriaceae isolates to analyse cephalosporin-resistance. We found that 82 out of 130 isolates were found to possess  $bla_{CTX-M}$  genes belonging to genogroup1 and they gave a hybridization profile corresponding to CTX-M-15 (68).

## Mobilization of $bla_{CTX-M}$ -genes

Recently, it has been observed that  $bla_{CTX-M}$  genes were mobilized to other bacterial genera. This seems to have occurred by recombinational events mediated by CR elements, *ISEcp1*, phage related elements, integrons and plasmids (6,12,73-77). *IS26* has

also been found to take part in mobilization of  $bla_{CTX-M-15}$  gene and in our recent studies we noticed a high occurrence and diverse insertion-positions in Indian bacterial isolates (6,14).

One study showed a high frequency (31%) of co-detection of *IS26* with  $bla_{CTX-M-15}$  (14). In that study, we observed amplified fragments of variable sizes (~650bp, ~700bp, ~900bp, ~950bp) but on sequencing, exact length of fragment was determined and it was found that the fragment 863bp (~900bp) was the most frequent *IS26* fragment in Indian Enterobacteriaceae isolates (14). On sequence analysis, insertion of *IS26* could precisely be detected for three different fragments (~650bp, ~700bp, ~900bp) at three variable positions within the *tnpA* gene, a transposase-coding gene of *ISEcp1* (Figure 2). Probably, *IS26* insertion into *tnpA* of *ISEcp1* inhibits further *ISEcp1*-mediated  $bla_{CTX-M-15}$  mobilization, and hence effectively traps  $bla_{CTX-M}$  onto plasmids, ensuring its maintenance in the gene pool (14). Recently, we observed *IS26* insertion in 48.2% of  $bla_{CTX-M}$  harbouring isolates of *Citrobacter* spp (6). In contrast to our previous finding (14) no diversity in the insertion points of *IS26* elements was noticed in those *Citrobacter* spp. and all isolates produced an amplicon of 400bp, similar to the clone A that was predominant in isolates from the United Kingdom (6).



**Figure 2.** Schematic diagram showing the insertion positions of *IS26* into *ISEcp1* for the ~650bp, ~700bp and ~850bp amplicons from Indian strains MS01, MS03 and MS02 respectively.

## AmpC $\beta$ -lactamases: the existing burden

AmpC  $\beta$ -lactamase, an Ambler class C  $\beta$ -lactamase, is an enzyme that confers resistance to both oxyimino- and 7- $\alpha$ -methoxycephalosporins, and monobactams (1). Although carbapenems are active against strains that harbour these enzymes, emergence of dual resistance has been described (2). AmpC  $\beta$ -lactamases are produced by numerous pathogens (78) and are emerging as an increasing cause of resistance in Enterobacteriaceae (14). These class C  $\beta$ -lactamases have now been discovered worldwide, including in India (4,79,80). Interestingly, increasing frequency of co-existence of  $bla_{ampC}$  with  $bla_{CTX-M}$  has also been reported recently in Enterobacteriaceae (4,5) as well as in *Citrobacter* spp. (6).

## History, chronology, and epidemiology of AmpC $\beta$ -lactamases

Chromosomal AmpC  $\beta$ -lactamases have been described in numerous bacterial pathogens, including *Escherichia coli*, *Enterobacter* spp., *Citrobacter freundii*, *Acinetobacter* spp., *Aeromonas* spp., *Proteus rettgeri*, and *Pseudomonas aeruginosa* (80). A chromosomal ampC gene is usually lacking in *Klebsiella* spp. and *Proteus mirabilis* (81). The first report regarding the transfer of cefoxitin-resistance was provided by Knothe *et al.* (82) in 1983 where they described the transfer of the resistance from *Serratia marcescens* to *Proteus* or *Salmonella* spp. but, the resistance segregated on transfer to *E. coli*. Subsequently, Bauernfeind *et al.* (83) described a *K. pneumoniae* isolate in 1989 that could transfer resistance to cefoxitin and cefotetan as well as to penicillins, oxyimino-cephalosporins, and monobactams to *E. coli*. The enzyme, termed CMY-1 for its cephamycinase activity, had an isoelectric point (pI) of 8.0 and was more sensitive to inhibition by sulbactam than by clavulanate or tazobactam, suggesting that it might be a class C enzyme. In 1990, Papanicolaou *et al.* (84) provided the first evidence that a class C  $\beta$ -lactamase had been captured on a plasmid. They described transmissible resistance to  $\alpha$ -methoxy- and oxyimino-  $\beta$ -lactams mediated by an enzyme (MIR-1) which possesses biochemical properties of a class 1  $\beta$ -lactamase. Subsequently, plasmid-mediated class C  $\beta$ -lactamases have been discovered worldwide. A recent

review of the epidemiology of AmpC enzymes provides a useful overview of this field, including the bacterial species from which they have been isolated, accession numbers, country of origin, and chronology of discovery. (80)

Plasmid-mediated AmpC (pMAmpC)  $\beta$ -lactamases have now been identified from around the globe, including Asia, Africa, Europe, the Middle East, North-, South-, and Central America. Moreover, several geographic clusters have been described including a North American cluster of MIR-1 and ACT-1, a Central and South American cluster of FOX-1 and FOX-2, and an Asian cluster of CMY-2, CMY-2b, LAT-1 and LAT-2 (80,85).

### Nomenclature of AmpC $\beta$ -lactamases

As shown in Table 1, class C  $\beta$ -lactamases have been named according to resistance to particular antibiotics, their inhibition by particular  $\beta$ -lactamases; the site of discovery; or even after the name of a patient.

**Table 1. Nomenclature for AmpC  $\beta$ -lactamases**

Criteria	AmpC-nomenclature
<b>Resistance to antibiotics</b>	
Cephamycins	CMY
Cefoxitin	FOX
Moxalactam	MOX
Latamoxef	LAT
<b>Type of <math>\beta</math>-lactamases</b>	
AmpC type	ACT
Ambler class C	ACC
<b>Site of discovery</b>	
Miriam Hospital in Providence Rhode Island, USA	MIR-1
Dhahran Hospital, Saudi Arabia	DHA
<b>Name of the patient</b>	BIL-1

### Genetic environment of $bla_{ampC}$ genes

Gene cassettes are responsible for the transfer of Ambler class A, B, and D  $\beta$ -lactamases; however, for class C  $\beta$ -lactamases, integrons have been implicated in the horizontal transfer (86). For the gene cassettes, a downstream 59-base element has been reported to act as a recombination site for incorporation into integrons (87). However, analyses of the  $bla_{ampC}$  sequences have revealed that AmpC-genes on plasmids are not linked to 59-base elements (80). Majority of the *amp*- genes have been described in a particular class 1 integron containing two copies of the 3' conserved segment (3'-CS1 and 3'-CS2) surrounding a common region (CR) and an antibiotic resistance gene (9,88). Apart from the mobile elements, such as the transposons or integrons, the genes for AmpC-enzymes have also been located on plasmids (87,89, 90). These mobile elements often carry multiple other resistance genes for various other antibiotics classes (2,22,89).

### Detection of AmpC-producers

It is unfortunate that the current Clinical and Laboratory Standards Institute (CLSI) documents do not indicate unambiguous screening and confirmatory tests that should be used for the detection of AmpC  $\beta$ -lactamases. Cefoxitin-resistance is often considered as the indicator for AmpC production and a zone of inhibition of 18 mm or less is considered a potential indicator for the presence of AmpC enzymes. A screen-positive bacterial isolate (based on cefoxitin disc susceptibility result) should then be confirmed by phenotypic "three-dimensional extract test" (TDET) as described originally by Coudron *et al.* (91).

We screened *Pseudomonas aeruginosa* isolates from burn patients and described the prevalence of AmpC  $\beta$ -lactamase (20% AmpC-producers and 10% AmpC-intermediate type) based on phenotypic detection methods. We also observed a high resistance (56.7%) to amikacin (92). This was the first report from India describing

the emergence of plasmid-mediated resistance to amikacin and occurrence of AmpC  $\beta$ -lactamase in *P. aeruginosa* strains. Subsequently, as developed for ESBL detection, we also modified the original TDET for AmpC detection and reported for the first time a user-friendly protocol for phenotypic detection of AmpC enzymes (93). Since its description in 2004, we have used our modified TDET and continue to get promising results for phenotypic AmpC detection (4). In an extensive analysis of the phenotypic and genotypic methods for AmpC detection, we reported that a cefoxitin zone diameter of 14 mm or less is a better indicator for describing presence of  $bla_{ampC}$  as a resistance mechanism, than a zone diameter of 18 mm as reported in earlier studies (4).

Amongst other phenotypic tests, Black *et al.* (94) found that an AmpC disk test was a simple, convenient and accurate means of detection of pMAmpCs in organisms lacking a chromosomal AmpC  $\beta$ -lactamase. Another method was developed by Yagi *et al.* (95) that involve augmentation of the inhibition zone around ceftazidime and cefotaxime discs by boronic acid. Later in 2006, Jacoby *et al.* (96) designed a disc based method that could be used to detect various  $\beta$ -lactamases in *E. coli* and *K. pneumoniae*.

### Genotypic detection of $bla_{ampC}$

Multiplex PCR has also been used for genotypic detection of AmpC  $\beta$ -lactamases, eg. Perez-Perez & Hanson (97). Among Indian Enterobacteriaceae, we observed  $bla_{ampC}$  of CIT family in 20% of *E. coli* and *Klebsiella* isolates. On sequencing of the representative isolates, CMY-6 AmpC  $\beta$ -lactamases was reported for the first time from India (4). Recently we have made some modifications to the original method of Perez-Perez and Hanson (97) and we noticed some interesting and novel findings not described in the original paper of Perez-Perez and Hanson (data not shown).

Lee *et al.* (98) developed DNA chips for simultaneous detection of various  $\beta$ -lactam-resistance genes. Furthermore, in 2007, Zhu *et al.* (99) developed a more advanced Multiplex Asymmetric PCR (MAPCR) based on an Oligonucleotide Microarray for detection of ESBLs and pMAmpC  $\beta$ -lactamase genes in Gram-negative bacteria. For further details on diagnostic procedures, readers are encouraged to refer to Jacoby (79) and Shahid *et al.* (80).

### Co-existence of CTX-M type $\beta$ -lactamases and other $\beta$ -lactamase enzymes with special reference to AmpC $\beta$ -lactamases

In recent years, multidrug resistance has increased in members of Enterobacteriaceae and plasmid-mediated  $\beta$ -lactamases (CTX-M and AmpC  $\beta$ -lactamases) were found as major contributors (100). Reports of multiple  $\beta$ -lactamases in a single bacterial strain are increasing for the Enterobacteriaceae especially in *K. pneumoniae* (101-104). We performed a systematic analysis and described the genetic environment of  $bla_{CTX-M}$ -producing *E. coli* and *Klebsiella* isolates. Co-occurrence of  $bla_{CTX-M}$  and  $bla_{ampC}$  was found in 77.3% isolates (4, 14). Recently we described for the first time the simultaneous occurrence of multiple  $bla$  genes (including  $bla_{CTX-M}$ ,  $bla_{TEM}$ ,  $bla_{SHV}$  and  $bla_{ampC}$ ) in class 1 integrons from *Citrobacter* spp. (6). In that collection of Indian *Citrobacter* spp.,  $bla_{CTX-M}$ ,  $bla_{TEM}$ ,  $bla_{SHV}$  and  $bla_{ampC}$  was noticed in 67.5%, 40%, 25%, and 40% isolates, respectively. Alarming, some isolates demonstrated simultaneous occurrence of insertion sequences *IS26* and *Orf513*, reflecting the complex evolution of resistance mechanisms. Our recent report also indicates that the genetic environment of  $bla_{CTX-M}$  has changed through insertion of *IS26* and *Orf513* (6).

### Newer $\beta$ -lactams (cephalosporins and carbapenems) and $\beta$ -lactamase-inhibitors in clinical practice

Since the discovery of penicillin by Alexander Fleming in 1928 from the mould *Penicillium*, numerous  $\beta$ -lactam antibiotics have been discovered (1,105). However, the pace of drug discovery has slowed down and few novel antibacterial agents have been discovered in recent years. Two novel  $\beta$ -lactam antibiotics, ceftobiprole and ceftaroline, have generated particular interest with studies demonstrating their potential as promising antimicrobial agents.

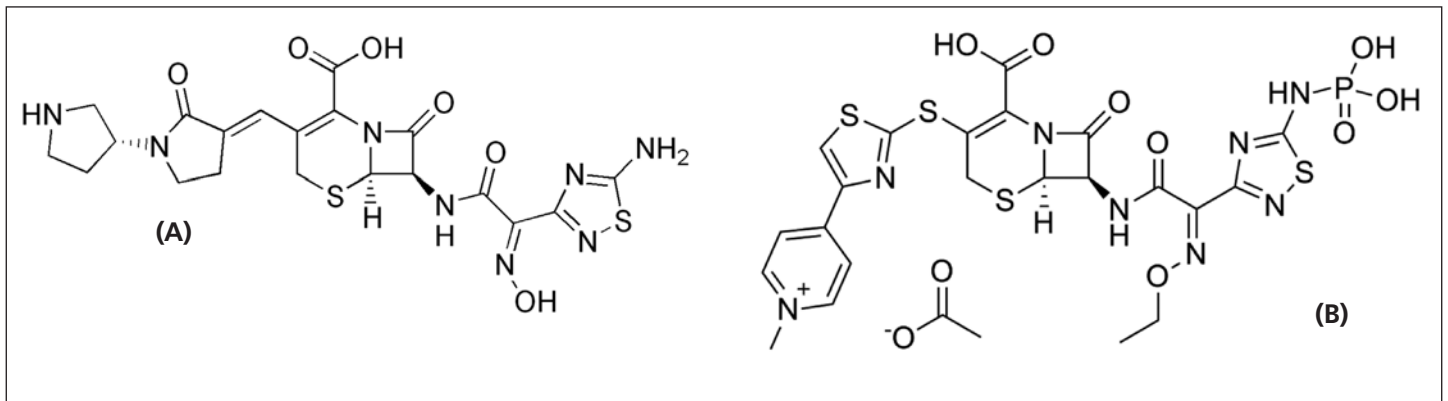


Figure 3. Chemical structures of newer cephalosporins, ceftobiprole (A) and ceftaroline fosamil (B).

### Ceftobiprole

Ceftobiprole is a pyrrolidinone -3-ylide-methyl cephalosporin and was formerly designated as BAL 9141/RO 63-9141 (Figure 3A). Ceftobiprole is active against methicillin-resistant *Staphylococcus aureus* (MRSA) due to a combination of potent binding to PBP2a and stability to Staphylococcal-penicillinase (1,106-109). However, its activity has also been reported against Enterobacteriaceae (106,109,110) and *Pseudomonas aeruginosa* (109,110). It was also found effective against ceftriaxone-resistant *Streptococcus pneumoniae* in a murine model of pneumonia (111) and against multiple Gram-negative and Gram-positive bacteria in a murine model of septicemia (106).

Previous studies demonstrated that ceftobiprole has an MIC<sub>90</sub> of between 1 and 4 mg/L against MRSA and methicillin-resistant coagulase-negative staphylococci (MRCoNS) (106,112). Studies involving serial passage of MRSA and MRCoNS in the presence of ceftobiprole showed that acquired resistance was slow to develop. One study observed a 4-fold increase in MIC to 8mg/L (107). Although in another study, the MIC of ceftobiprole never raised more than one-doubling dilution after serial passage (106). In another study, slightly higher MIC<sub>90</sub> (4 mg/L for *E. cloacae* and 1 mg/L for *Citrobacter* spp.) were observed against non-derepressed AmpC producing *E. cloacae* isolates and *Citrobacter* spp. when compared to that of cefepime (0.5 mg/L for both *E. cloacae* and *Citrobacter* spp.). However, the MIC<sub>50</sub> of these agents was found to be comparable (112).

As with other cephalosporins, the *in vitro* activity of ceftobiprole against ESBL-positive strains of *E. coli*, *K. pneumoniae* and *P. mirabilis* was found to be diminished (112). This observation reflects the increased susceptibility of ceftobiprole and other cephalosporins

to hydrolysis by class A (mainly TEM and SHV) β-lactamases. A high MIC<sub>90</sub> of ceftobiprole (> 32 mg/L) was observed among the putative CTX-M isolates. It has been suggested that decreased activity of ceftobiprole relative to ceftazidime against ESBL-producers is due to the instability of ceftobiprole to CTX-M β-lactamases (112). Pillar *et al.* (112) also observed that activity of ceftobiprole was comparable to cefepime against Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* spp. and it displayed potent activity against MRSA.

Silva *et al.* (113) observed that *Enterococcus faecalis* isolates were inhibited by ≤ 4mg/L of ceftobiprole, while cefepime showed lesser antibacterial activity (MIC<sub>90</sub> > 128 mg/L). In contrast, MICs of both of these cephalosporins for *E. faecium* were high (ceftobiprole MIC<sub>90</sub> = 128 mg/L and cefepime MIC<sub>90</sub> ≥ 128 mg/L). Activities of ceftobiprole and cefepime were similar against non-ESBL-producing *E. coli*. Against ESBL producers, the activity of both ceftobiprole (MIC<sub>90</sub> = 128 mg/L) and cefepime (MIC<sub>90</sub> of 64 mg/L) was reduced. Ceftobiprole showed good activity against MRSA (MIC<sub>50</sub> = 0.5 mg/L ;MIC<sub>90</sub> = 2 mg/L) and methicillin-sensitive *Staphylococcus aureus* (MSSA; MIC<sub>50</sub> = 0.25 mg/L; MIC<sub>90</sub> = 0.5 mg/L) (113). However, cefepime showed poor activity against MRSA and MSSA (MIC<sub>90</sub> 16-fold higher than that of ceftobiprole). In another study, ceftobiprole was evaluated for its activity both against Gram-positive and Gram-negative organisms. The MIC<sub>90</sub> of ceftobiprole against various organisms was lower (MIC<sub>90</sub> 0.5 mg/L) than that of as compared to 1mg/L (MIC<sub>90</sub> of other three comparator drugs viz. vancomycin, teicoplanin and linezolid). However, all four drugs have similar MIC<sub>90</sub> of 2 mg/L against MRSA. It showed 4-fold better MIC<sub>90</sub> (0.5mg/L) against MSSA (114). In conclusion, the broad-spectrum bactericidal activity of ceftobiprole suggests its potential use in the treatment of serious infections (114).

## Ceftaroline

Ceftaroline fosamil (Figure 3B) was formerly known as PPI-0903 and TAK-599 (115,116). Ceftaroline possesses broad-spectrum activity that encompasses pathogens associated with skin infections such as MRSA,  $\beta$ -haemolytic Streptococci, *E. faecalis* and most Gram-negative bacilli that are ESBL non-producers. MRSA and MRCoNS were reported to be susceptible to ceftaroline with an MIC<sub>90</sub> ranging between 0.25mg/L to 2 mg/L (115,116). There are reports describing its potent activity against vancomycin-resistant MRSA strains, multidrug-resistant *S. pneumoniae* strains, and *Haemophilus influenza* (117,118). Its activity against MRSA is attributed to its increased affinity for modified PBP2a. It has been predicted by recent pharmacokinetic and pharmacodynamic target attainment studies that 600 mg of ceftaroline every 12 hours would provide %T> MIC [% of time the drug concentration remains above the

MIC] target attainment for key skin pathogens with ceftaroline MICs of  $\leq$  2 mg/L (119). Ceftaroline possess improved anti-MRSA activity as a result of the altered 3' side chain that interacts with an allosteric binding site on PBP2a, allowing the  $\beta$ -lactam ring to have optimal access to the active-site serine, acylating the site and initiating the series of biochemical events that result in bacterial cell death (120). In those studies, clinical trial data was found to be in excellent concordance with pharmacodynamic analyses (120) supporting the promising potential of ceftaroline to be used in current clinical practice.

Salient features of some of the established- or developmental-phase cephalosporins, carbapenems, and  $\beta$ -lactamase inhibitors are shown in Tables 2, 3 and 4, respectively.

**Table2. Some of the newer cephalosporins and their salient features/activities.**

Newer cephalosporins	Salient features/ activities	References
S-3578	- activity against MRSA and <i>Pseudomonas aeruginosa</i> , also a promising newer cephalosporin for polymicrobial infections or superinfection caused by Gram-positive and Gram-negative bacteria; this compound does not appear to be in active development.	121; 122
BMS-247243	- useful in treatment of infections caused by MRSA, <i>Strptococcus epidermidis</i> , <i>S. hemolyticus</i> . -MIC <sub>90</sub> for methicillin-sensitive Staphylococci ranged from <0.25 to 1 mg/L. -MIC <sub>90</sub> for <i>S. aureus</i> was 4-fold higher than $\beta$ -lactamase non-producing strains. - has improved affinity for PBP2a and affinity for PBP2a was >100 fold better than methicillin or cefotaxime.	123
RWJ-54428	-active against Gram-positive organisms including MRSA and drug resistant Enterococci; has affinity for PBP2a and PBP5.	124

**Table 3. Some of the newer carbapenems and their salient features/ activities.**

Newer carbapenems	Salient features/ activities	References
<b>Doripenem</b> (formerly known as S-4661)	-showed potency against Gram-positive cocci similar to that of imipenem or ertapenem, and activity against Gram-negative similar to meropenem but 2-or 4-fold superior to imipenem.	125; 126; 127
<b>Ertapenem</b> (formerly known as MK-0826 and L-749,345)	-active against Enterobacteriaceae, Gram-positive organisms and anaerobes but less active against non-fermenters. -binds more strongly to PBP2a of <i>E. coli</i> as compared to PBP3 and has good affinity for PBP1a and PBP1b. -in contrast to imipenem, it binds primarily to PBP-2 than PBP1a and 1b.	128; 129
<b>trans-3,5-disubstituted</b>	-these agents are 32-64 fold more potent than imipenem. Although these were less active <i>in vitro</i>	130
<b>Pyrrolidinylthio-1 -methyl carbapenems</b> (J-111, 347; J-111, 225; J-114, 870; J-111, 347)	-against MRSA and MRCoNS than vancomycin. -active against Gram-positive as well as Gram-negative organisms including <i>P. aeruginosa</i> -MRCoNS were inhibited by J-111,347 (prototype) at concentrations of 2mg/L. -MIC <sub>90</sub> of J-111, 225 against MRSA & MRCoNS was reported as 2mg/L. -MIC <sub>90</sub> of J-114, 870 and J-111, 347 against MRSA & MRCoNS was 4mg/L.	
<b>2-(Thiazol-2-ylthio)-1 -methyl</b>	-SM-17466 and its derivative show potent anti-MRSA activity corresponding to high affinity for PBP2a.	131; 132
<b>Carbapenems</b> (SM-197436; SM-232721; SM-232724)	-among Gram-negative these carbapenems were found highly active against <i>H. influenzae</i> , <i>M. catarrhalis</i> , <i>B. fragilis</i> . -showed activity equivalent to imipenem for <i>E. coli</i> , <i>K. pneumoniae</i> and <i>Proteus</i> spp. -although it showed good activity against MRSA, but did not show activity against <i>E. faecium</i> . -SM-232721 and SM-232724 found active against MRSA and MRSE with MIC <sub>90</sub> of ≤4 mg/L. -MICs of SM-19746, SM-232721 and SM-232724 for streptococci including penicillin-resistant <i>S. pneumoniae</i> strains ranged from <0.063 to 0.5 mg/L.	
<b>CS-023 (RO4908463)</b> (formerly known as R-115685)	-binds with high affinity to PBP1, PBP2, PBP4 of <i>S. aureus</i> . -binds to PBP1b, PBP1b, PBP2 & PBP3 of <i>E. coli</i> . -more stable to hydrolysis by human renal peptidase-I than meropenem or imipenem. -bactericidal against <i>P. aeruginosa</i> & MRSA, and also showed broad-spectrum activity against Gram-positive and Gram-negative bacteria including penicillin-resistant <i>Streptococcus pneumoniae</i> , <i>H. influenzae</i> , and members of Enterobacteriaceae.	1
<b>Faropenem medoxil</b> (Formerly, Faropenem daloxate)	-active against non-penicillin susceptible <i>S. pneumoniae</i> and <i>M. catarrhalis</i> . -active against non-penicillin susceptible <i>S. pneumoniae</i> and <i>M. catarrhalis</i> . -active against <i>S. pneumoniae</i> with MIC <sub>90</sub> of 0.5mg/L and >4 fold more active than cefdinir & cefuroxime (MIC <sub>90</sub> , 4mg/L).	133
<b>Tebipenem</b> (Formerly, LJC11,036)	-showed stability to dehydropeptidase-I. -showed potent activity against various microorganisms, except MBL-producing pathogens and MRSA strains	134
<b>ME 1036</b> (Formerly known as CP5609)	-showed excellent activity against MRSA and MRCoNS. -also showed potent activity against penicillin-resistant <i>S. pneumoniae</i> , <i>E. faecalis</i> , <i>H. influenzae</i> , and members of Enterobacteriaceae. -slightly less active than imipenem and meropenem in presence of AmpC β-lactamase.	117
<b>SMP-601</b> (Formerly PZ-601)	- high affinity for PBP and hence showed enhanced activity against MDR Gram-positive and Gram-negative organisms including MRSA and <i>Pseudomonas</i> spp. -more active against ESBL-producing <i>E. coli</i> and <i>K. pneumoniae</i> .	135
<b>Trinemis</b> (Sanfetrinem; Formerly known as GV104326)	-have carbapenem related structure but with cyclohexane ring attached across carbon1 & 2. -sanfetrinem was found susceptible to TEM-1 & TEM-10 enzyme. -like imipenem and cefixime, but unlike cefpodoxime, it retained activity against <i>P. vulgaris</i> & <i>K. oxytoca</i> that hyperproduced chromosomal class A β-lactamase.	136

**Table 4. Established and newer  $\beta$ -lactam inhibitors and their salient features/ activities.**

$\beta$ -lactam inhibitors	Salient features/ activities	References
<b>Tazobactam</b>	- inhibit action of bacterial $\beta$ -lactamase. -exhibit 10 times greater activity than clavulanic acid against CTX-M $\beta$ -lactamase.	137
<b>Sulbactam</b>	-exhibit some of the properties as clavulanic acid. -cephalosporins were inhibited less effectively than penicillinases or broad-spectrum $\beta$ -lactamases. -was slightly more active against cephalosporinase as compared to clavulanic acid.	1
<b>Clavulanic acid</b>	-is first suicide inhibitor of $\beta$ -lactamase, and was isolated from <i>Streptomyces clavuligerus</i> . -showed activity against broad-spectrum $\beta$ -lactamase producing <i>K. pneumoniae</i> .	138
<b>Oxapenem</b>	-were found to be potent $\beta$ -lactamase inhibitor but have poor stability. -reduce MICs for ceftazidime against class C hyperproducing, TEM, and SHV derived ESBL producing strains however these compounds failed to enhance activity of ceftazidime against derepressed Amp C producing <i>P. aeruginosa</i> strains.	139
<b>NXL104 (Formerly AVE1330A)</b>	-inhibits both class A and C $\beta$ -lactamase. -in combination with ceftazidime, it exhibit broad-spectrum activity against class A- and C-producing members of Enterobacteriaceae. -was found effective in restoration of activity of ceftazidime, cefotaxime or imipenem against class A carbapenemase-Producers (IMI-1, NMC-A, GES-2, GES-3, GES-4, KPC-2 and KPC-3). -activity of ceftazidime/NXL 104 against KPC-2 or KPC-3 enzymes was noticeable with MIC $\leq$ 0.015 to 0.5 mg/L as compared to 64 to >128 mg/L.	140; 141
<b>Mono- and bicyclic-bridged</b>	-are stable to hydrolysis by class B MBLs and many class D $\beta$ -lactamases.	142; 143
<b>Monobactams (BAL0019764; BAL0030072; BAL30376)</b>	-reacted with AZT with AmpC and ESBL enzymes and resisted attack by metallo- $\beta$ -lactamases. -IC <sub>50</sub> of BAL 30072 was 0.09 $\mu$ M while IC <sub>50</sub> of clavulanate was >100 $\mu$ M against AmpC enzyme from <i>C. freundii</i> . -BAL30376 is a combination of BAL0019764, BAL0029880 and clavulanic acid in the ratio of 1:1:1 by weight. - <i>In vitro</i> activity was observed against Gram-negative bacilli. -MIC <sub>90</sub> of BAL 30376 for <i>E. coli</i> , <i>K. pneumoniae</i> , and <i>E. cloacae</i> were 4, 1, and 4 mg/L, respectively, while MIC <sub>50</sub> were found to be 0.5, 0.5, and 1 mg/L for respective organisms. MIC <sub>50</sub> of 1-4 mg/L was observed against strains which had CMY-2, CTX-M, multiple combinations of SHV, CTX-M, CMY, OXA, and TEM-type enzymes, while meropenem, a most potent comparator drug, had MIC <sub>50</sub> of 0.03 mg/L. -against IMP containing Gram-negative bacilli, MIC <sub>50</sub> of BAL 30376 was 0.5-2 mg/L and that of meropenem was 2- $\geq$ 64 mg/L. MIC <sub>50</sub> of this inhibitor was 1-4 mg/L for VIM, GIM and SPM producers. -BAL 30376 was found potent inhibitor against IMP-4, IMP-13 or IMP-16, VIM-1, VIM-2, VIM-4, GIM-1 and SPM-1 producers.	
<b>LK-15</b>	-is a tricyclic carbapenem-inhibitor of serine $\beta$ -lactamases. -MIC <sub>90</sub> ranged between 16 and >128 mg/L for MRSA, penicillin-resistant <i>S. pneumoniae</i> , <i>B. fragilis</i> , <i>P. aeruginosa</i> and Enterobacteriaceae however, it was found to be $\leq$ 2 mg/L for MSSA, <i>S. pyogenes</i> , penicillin-susceptible <i>S. pneumoniae</i> and <i>H. influenzae</i> . -it decreased the MICs of aztreonam, ceftazidime, and cefuroxime for <i>B. fragilis</i> (8- to $\geq$ 128-fold) and $\beta$ -lactamase-producing members of Enterobacteriaceae (up to $\geq$ 64-fold).	144
<b>CP3242</b>	-is an MBL inhibitor and inhibits competitively both IMP-1 and VIM-2. -specifically lowered the MICs of $\beta$ -lactams against MBL-producing <i>E. coli</i> transformants. -significantly reduces the MICs of biapenem in a concentration dependent manner (2 to 32 mg/L) against MBL-producing <i>P. aeruginosa</i> . -MIC <sub>90</sub> of biapenem was lowered from 512 to 16 mg/L in the presence of 32 mg/L of CP3242.	145

## Conclusions and future strategies

There are established reports on the increasing frequency of occurrence of *bla*<sub>CTX-M</sub> and *bla*<sub>ampC</sub> in Indian Enterobacteriaceae (4,14) including *Citrobacter* spp. (6). Even more alarming is the simultaneous occurrence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>ampC</sub> along with *bla*<sub>CTX-M</sub> on class 1 integrons in *Citrobacter* spp. (6). The apparent acquisition of IS26 and ORF513 on integrons and plasmids in Indian *Citrobacter* strains is also startling. This strongly suggests a 'turnover' of these mobile elements (IS26 and ORF513) in the population over time and thus complicates the resistance problem. We are aware that resistance to third and fourth generation cephalosporins has become a major concern worldwide and even more frightening is the recent emergence of carbapenem-resistance. There is an urgent need to implement strict infection control and antibiotics prescribing policies in India. Over the counter availability of antibiotics is still a common practice in India and this should immediately be stopped. As an immediate measure to combat the problem of resistance, combinations of cephalosporins with newer  $\beta$ -lactam inhibitors may be used as alternatives to reduce selection pressure. An alternative approach, which was also suggested in our earlier publication (1), could be periodical prescription rotation between  $\beta$ -lactam-aminoglycoside combination therapy,  $\beta$ -lactam- $\beta$ -lactamase-inhibitor combination, and the carbapenems.

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## Author information

Mohd. Shahid, MBBS MD PhD FNZIMLS, Associate Professor & Consultant Microbiologist

Department of Microbiology, JN Medical College & Hospital, Aligarh Muslim University, Aligarh-202 002, U.P., India. E-mail: shahidsahar@yahoo.co.in

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# An evaluation of laboratory methods for the diagnosis of toxigenic *Clostridium difficile* infection: enzyme immunoassays for glutamate dehydrogenase and toxins A and B, real time PCR (*tcdC* gene) and stool culture

Angela D Horridge, Mary F Nulsen and Rosemary Ikram

## Abstract

**Background:** *Clostridium difficile* is a major cause of health-care associated infections (HAI) and is the principle cause of diarrhoea in hospitalised patients. Enzyme immunoassays (EIAs) that detect *C. difficile* toxins A and B are commonly used by the laboratory for the diagnosis of toxigenic *C. difficile* infection (CDI). However, unacceptably low sensitivities and specificities for these toxin EIAs have been reported and attention has been directed at investigating more sensitive assays such as PCR and the use of algorithms for the detection of toxigenic *C. difficile* in order to improve the laboratory diagnosis.

**Objective:** To evaluate six tests for the laboratory diagnosis of *C. difficile* disease individually and in combination in terms of their sensitivity, specificity, negative predictive value, positive predictive value, timeliness and economy.

**Methods:** Diarrhoeal stool samples (n=346) were tested by a glutamate dehydrogenase, three EIAs for *C. difficile* toxins A and B, a real time (RT)-PCR to detect the *tcdC* gene and culture. The results of these assays were compared with a reference stool culture method using *C. difficile* agar followed by a toxin EIA on broth subcultures and the LightMix *C. difficile* RT-PCR for the *tcdC* gene on isolates subcultured on fastidious anaerobe agar. The performances of the individual assays and in combination were assessed and the results compared to reference culture.

**Results:** Fifteen specimens (4.3%) were considered positive for toxigenic *C. difficile* when compared to the reference standard. The sensitivities, specificities, positive predictive values (PPVs) and negative predictive values (NPVs) respectively were 86.7%, 96.7%, 54.2% and 99.4% for the Techlab C.DIFF Chek-60, 80%, 100%, 100% and 99% for the Techlab C. Diff Tox A/B, 80%, 98.2%, 66.7% and 99.1% for the Meridian Premier Toxin A/B, 80%, 99.7%, 92.3% and 99.8% for the Immunocard A/B and 93.3%, 99.7%, 93.3% and 99.7% for the LightMix *C. Difficile* RT-PCR

**Conclusions:** The *C. difficile* toxin A/B EIA kits failed to detect 3 of 15 true positive samples. The GDH EIA was not sensitive enough to be used as a screening assay in combination with any toxin A/B EIA. Combining the GDH EIA with any other test would increase workload, cost and turnaround time with no improvement in sensitivity. The best performing individual test in regards to sensitivity and turnaround time was the LightMix *C. difficile tcdC* RT-PCR. The best performing combination of assays was both a toxin A/B EIA on stool and culture. This would allow the reporting of 97.7% of results within 48 hours with 100% sensitivity and specificity.

**Key words:** *Clostridium difficile*, glutamate dehydrogenase, toxins A and B, real-time PCR, culture, algorithm, sensitivity, specificity, positive predictive value, negative predictive value.

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## Introduction

*Clostridium difficile* is a major cause of health-care associated infections and is the principle cause of diarrhoea in hospitalised patients, particularly in those with a recent history of antibiotic use (1). The organism is a spore-forming gram-positive anaerobic bacillus and pathogenesis is related to the production of toxin A, an enterotoxin and toxin B, a potent cytotoxin (2,3). *C. difficile* can cause a range of clinical symptoms from mild to severe diarrhoea and more seriously, pseudomembranous colitis, toxic megacolon, bowel perforation, sepsis and death (4).

The incidence of *C. difficile* infection has been increasing in individuals considered at-risk; i.e. individuals in health care facilities, those with a recent history of hospitalisation, prior use of antibiotics or chemotherapeutics, the elderly and immunocompromised (1,5). There are also reports of an increased incidence of *C. difficile* infection in populations previously considered to be low-risk, including children, pregnant women and previously healthy individuals with no known risk factors (4,6). Certain strains of *C. difficile* have also been associated with increased severity and mortality (7,8).

The economic burden of *C. difficile* infection is significant and in the United States it has been estimated that the overall annual cost of management of *C. difficile* infection exceeds \$1.1 billion dollars (9). The considerable costs associated with the management of *C. difficile* infection as well as the apparent increased incidence and severity of *C. difficile* infection has led to renewed interest in the laboratory diagnosis of the disease.

Laboratory tests available for the diagnosis of *C. difficile* infection include the cell cytotoxicity neutralisation assay (CCNA), culture, enzyme immunoassays (EIAs) and PCR. The majority of laboratories use EIAs to detect *C. difficile* toxins A and B as they are reasonably rapid and inexpensive (10,11). However, the value of toxin EIAs for diagnosis has been debated over the past few years due to reported low sensitivities and poor positive predictive values (12-17). This has led many to look at alternative methods to improve the laboratory diagnosis of *C. difficile* infection including highly sensitive PCR assays that target the toxin genes (mainly *tcdB* and *tcdC*) and the use of algorithms that utilise two or more diagnostic techniques (14,18,19).

The introduction of new, possibly more virulent *C. difficile* strains to New Zealand requires improved laboratory methods for *C. difficile* infection diagnosis. Incorrect laboratory results due to either lack of sensitivity or specificity can lead to inappropriate patient care with an increased possibility of cross-infections, subsequently increasing the financial burden on the health care system.

## Materials and methods

### Study description

Consecutive stool specimens were collected from March 2010 through to August 2010 from patients aged >20 years being investigated for the cause of their diarrhoea. Stools were excluded from the study if another pathogen was identified. All stool samples included in this study were unformed and contained a minimum of 2.0 mL of sample to enable all testing to be performed. All specimens included were kept at 2-4°C and were less than 48 hours old when they arrived at the laboratory. Specimens were divided into multiple vials and frozen at -70°C until testing. Specimens had all assays performed within 6 weeks of storage and were thawed only once before testing. There were two duplicate patient specimens and these were collected at least 13 days apart. Ethical approval was obtained for this project from the New Zealand Health and Disability Ethics Committee - Upper South A Region.

### Assays

All stools included in the study were tested for glutamate dehydrogenase (Techlab C.DIFF CHEK-60), *C. difficile* toxins A and B (Techlab C.DIFF TOX A/B II, Meridian ImmunoCard Toxins A/B, Meridian Premier Toxins A & B), and a real-time PCR assay that targets the *tcdC* gene (LightMix Kit *C. difficile*, TibMolBiol, distributed by Roche Diagnostics NZ) and by culture. All commercial EIAs and the RT-PCR were performed according to the manufacturer's instructions.

jars using anaerobic pouches (Anaero-Pack™, Mitsubishi Gas Chemical Company Inc. distributed by Ngaio Diagnostics) at 35°C for 48 hours. After 48 hours incubation, isolates resembling *C. difficile*, based on typical morphology and characteristic odour, were subcultured onto fastidious anaerobe agar (FAA, Fort Richard Laboratories Ltd, NZ) anaerobically for a further 48 hours at 35°C. Colonies were then checked for fluorescence under U.V. light and were gram stained. Isolates from FAA (three to five colonies) were sampled and stored for PCR and three to five colonies were subcultured in Brain-Heart Infusion broth (BHI, Fort Richard Laboratories Ltd, NZ) for 48 hours at 35°C anaerobically to allow for toxin production. The BHI broth supernatant was then tested for toxin A/B by the ImmunoCard Toxin A/B as per stool protocol. (NOTE: kit only validated by manufacturer for use on stools).

### Results

*C. difficile* was detected in 19 (5.5%) of 346 stools (Table 1). Fifteen (79%) were toxin producing strains and four (21%) were non-toxin producing strains. Overall, 4.3% of specimens were considered as true positives for toxigenic *C. difficile* when compared to the reference standard. The sensitivities, specificities, PPVs and NPVs are shown in Table 1.

**Table 1. Performance characteristics of four EIAs, a RT-PCR and culture used to detect *C. difficile* and *C. difficile* toxins A and B\***

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
C. DIFF CHEK-60 GDH (Techlab)	86.7	96.7	54.2	99.4
C.DIFF TOX A/B (Techlab)	80	100	100	99.1
Premier Tox A/B (Meridian)	80	98.2	66.7	99.1
ImmunoCard Tox A/B (Meridian)	80	99.7	92.3	99.1
LightMix <i>tcdC</i> PCR (TIBMOLBIOL)	93.3	99.7	93.3	99.7
Culture†	93.3	98.8	77.8	99.7

\* Results of 346 stools compared with the reference culture technique described in methods. † Stools ethanol shocked prior to culture on CDA followed by subculture onto FAA. PPV = positive predictive value, NPV = negative predictive value.

For the RT-PCR, a 1:3 dilution of the stool was made in STAR Buffer (Roche Diagnostics), thoroughly mixed by vortexing and then centrifuged for 1 minute at 3500rpm (550 x g). The supernatant was removed and frozen at -70°C until tested. Extraction for PCR was performed on 200 µL of supernatant by the MagNA Pure LC (Roche Diagnostics) using a Total Nucleic Acid Kit I, High Performance (Roche Diagnostics). For the PCR on cultured isolates, 3-5 colonies from fastidious anaerobe agar (FAA) subculture were placed into a vial containing 200µL PCR grade water, 180µL of MagNA Pure Bacterial Lysis Buffer (Roche Diagnostics) and 20µL of Proteinase K (Roche Diagnostics). Samples were vortexed and frozen at -70°C until testing and processed on the MagNA Pure LC as previously described. All extracted samples were run on the LightCycler 2.0 (Roche Diagnostics) in 20uL reactions for real-time amplification and detection. A non-template control (PCR grade water) and two positive controls that were provided with the kit (wild-type and 18bp del. mutant) were included with each batch.

### Culture

Thawed stools were mixed with 95% ethanol in a 1:1 ratio and incubated at room temperature for 30 minutes (20). Four drops (200µl) of diluted stool was plated onto *C. difficile* selective media (CDA, Fort Richard Laboratories Ltd, NZ). This media is supplemented with 8 mg/L cefoxitin, 250 mg/L D-cycloserine and 5% defibrinated horse blood. Plates were incubated in anaerobic

Of the 15 toxigenic isolates, 10 stools tested positive by all assays, one stool tested positive by all assays except culture and this was considered as a true positive for the purpose of calculations. On repeat culture, *C. difficile* was isolated from this specimen. Two stools tested positive by culture and PCR and negative by all other assays. One stool tested positive by culture, PCR and GDH only. One stool tested positive by all assays except PCR.

One sample was positive by the PCR assay alone and this was considered a false positive as *C. difficile* was not isolated by culture. There was one false positive toxin result by the ImmunoCard Tox A/B and six false positives for the Meridian Premier Tox A/B kit. There were 16 initially invalid ImmunoCard A/B assays due to blockage of the membrane. These were negative when a new dilution was made and centrifuged in 1.5mL eppendorf tubes for 10 seconds (at 550 x g) prior to testing. Although not specifically recommended by the manufacturer to avoid blockages, this was done to obtain a valid result. Eleven positive GDH results were considered as false positives; culture was negative for eight of these and non-toxigenic *C. difficile* isolates were cultured from three. There were three false negative GDH results (one non-toxigenic isolate and two toxigenic isolates were cultured). All true positive samples that tested positive by toxin A/B EIA also tested positive by the GDH assay. The performance of the two and three step algorithms are compared in Table 2.

**Table 2. Performance of two-step and three-step algorithms for detecting toxin-producing *C. difficile* compared to reference culture**

Algorithm	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
GDH + Techlab Tox A/B	80	100	100	99.1
GDH + Prem. Tox A/B	80	100	100	99.1
GDH + I/Card A/B	80	100	100	99.1
GDH + PCR	80	100	100	99.1
GDH + Toxigenic Culture	86.7	100	100	99.4
GDH + Techlab Tox A/B + PCR	86.7	100	100	99.4
Culture + ImmunoCard Tox A/B on isolates	93.3	100	100	99.7
Culture + PCR on isolates*	93.3	100	100	99.7
Techlab Toxin A/B (stool) and Culture (+/- toxin testing of isolate)†	100	100	100	100

\* One toxigenic *C. difficile* isolate was repeatedly inhibited in PCR and was excluded from the calculation. † Performing both stool toxin EIA and culture as routine on all stools and performing a toxin assay on the isolate only if stool toxin is negative.

### Culture

Of the 15 toxigenic isolates, 13 tested positive by both the RT-PCR (isolates from FAA) and the ImmunoCard toxin A/B assay (broth culture). One isolate was positive by PCR but negative for toxin on broth. On repeat broth culture this tested positive for toxin. One toxigenic isolate tested positive for toxins A and B in broth but the RT-PCR was repeatedly inhibited. Two broths were repeated as they failed to grow initially.

### Discussion

The measured sensitivities of the *C. difficile* toxin A/B EIAs and the GDH EIA in our study were similar to those reported by Eastwood *et al* (16) when compared to toxigenic culture (Table 1). All three toxin A/B EIAs failed to detect toxin in three of 15 true positive stools (the same specimens) and of these, GDH was not detected by EIA in two. These two samples were positive by PCR and culture only, suggesting that these may have been early infections where the better sensitivity of culture and PCR were able to detect *C. difficile* sooner than the less sensitive EIAs. One patient had an additional stool submitted two weeks later and all assays were positive at this time. The LightMix *tcdC* PCR and the culture method used were the best performing single tests, although the PCR assay has a much faster turnaround time with results available within hours. In the case of culture, negative cultures can be reported within 48 hours, while positive cultures could take up to 6 days to confirm.

The PPVs of the C.DIFF TOX A/B, the ImmunoCard A/B and the LightMix PCR in our study were higher than expected, despite a low number (4.4%) of toxigenic *C. difficile* (Table 1). The PPV of the Premier Tox A/B kit was lower than the other two toxin EIAs (66.7%) due to the six false positive results, results similar to other reports (21).

There have been contradictory reports on whether the GDH assay is sensitive enough to be used as a screening assay. The Techlab C. DIFF CHEK-60 GDH EIA used in our study has been shown in various reports to be an excellent screening assay with sensitivities approaching 100% and high NPVs (14,17,22-24), while other reports have found significantly lower sensitivities (16,25). The sensitivity of the GDH assay in our study was only 86.7%, similar to those reported by Eastwood *et al* (16) and Larson *et al* (25), suggesting that it is not suitable as a screening assay, missing approximately 15% of CDI cases.

The LightMix *C. difficile* PCR detects both the wild-type and *tcdC* 18bp deletion. No strains with an 18bp deletion were detected in our study. We detected 14 of 15 true toxigenic *C. difficile* cases on

stool with one false positive result giving a sensitivity of 93.3% and a specificity of 99.7% (Table 2), similar to other reports of commercially available molecular methods (16,19,5). Our results were slightly better than those obtained by Sloan *et al* (15) using a PCR that targets the *tcdC* gene (wild type and the 18bp deletion mutant) and performed on the Roche LightCycler™. They found a sensitivity of 86% and a specificity of 97% compared to a reference standard of culture and PCR to detect the toxin genes. Six culture positive specimens were negative by PCR with the authors suggesting that the efficiency of nucleic acid extraction or sampling may have been the cause (15). As the false negative PCR in our study was re-extracted and repeated by PCR and remained negative (internal control returned a positive result), this was unlikely to be the cause of the PCR failure. It is possible that certain substances in stool samples may interfere with the PCR reaction but as there was no inhibition of the reaction, it is unclear why the PCR did not detect the organism. It may also be possible that this strain has a deletion in the target gene, *tcdC* interfering with primer binding. When the cultured *C. difficile* isolate from this same patient was tested by PCR it was inhibited (the internal control gave a negative result). The sample was re-extracted and the PCR repeated as before and also in a 1:3 dilution but it remained inhibited. All four EIAs performed on this stool sample were positive and broth culture tested positive by toxin A/B EIA.

There was one false negative culture in our study and when culture was repeated, toxigenic *C. difficile* was isolated. All other assays were positive; therefore the sample was considered a true positive despite the initial failure of culture. It is unknown why culture failed but, as this specimen was noted to be particularly mucoid, perhaps the organism was unevenly distributed in the stool.

As the specificity of the GDH assay is low, this assay must be used in combination with a toxin detection assay. In our study there was no advantage in the use of a two-step algorithm using the C.DIFF CHEK-60 (GDH) EIA as a screen followed by a toxin A/B EIA as a confirmatory assay as the sensitivity of the GDH assay was only slightly better than that of the toxin EIAs. If a GDH assay was used as a screening assay it would incur a significant cost to the laboratory as well as increase the workload, although it would have improved the PPV of the Premier Tox A/B EIA due to the six false positive samples found with this assay. Twelve samples that were tested by both the GDH and the toxin A/B assays were GDH positive but toxin A/B negative. These samples would require additional testing due to the low sensitivity of the toxin assays. If PCR was the third step, only one additional sample would have been determined as being a true positive. Twelve others would have been classified as

true positives as they were both GDH and toxin positive and the remainder were GDH negative. More than 24 additional tests would have been performed on this group of 346 samples. An algorithm including GDH as a screen could have been useful if there were a lot of false positive toxin A/B EIA results and poor PPVs, as seen in other reports (29). This was not seen in the population tested here, despite a low number (4.3%) of toxin-producing *C. difficile*. Novak-Weekley *et al* (19), using toxigenic culture as a gold standard, also found no improvement in sensitivity using a two-step algorithm (GDH and toxin A/B EIA) over the toxin A/B EIA alone. They found a sensitivity of only 55.6% for the two-step and a sensitivity of 58.3% for the individual toxin assay. There was an improvement in sensitivity to 83.1% when CCNA was included as a third step. In contrast, others have found significant improvement in results for similar two-step algorithms, i.e., a GDH assay followed by a toxin A/B EIA) over the use of a toxin assay alone (17).

A possible limitation of our study was the low sample size and low prevalence of toxigenic *C. difficile*. The majority of patients included in our study were considered low-risk for CDI (only 70 of 346 had *C. difficile* testing specifically requested). The remaining 275 samples were mainly from community patients being investigated for the cause of their diarrhoea and no toxigenic *C. difficile* was found in this group. Three of the four non-toxigenic strains found in this study were harboured by 'low-risk' patients and one was isolated from a patient suspected of having CDI.

Individual laboratories need to determine what test or algorithm is both practical and feasible for them to perform. An ideal algorithm or test would need to balance turnaround time, cost, sensitivity and specificity, NPVs and PPVs against available resources and it is likely that different algorithms may be necessary depending on requestor testing patterns and the prevalence of CDI in the population being tested (27). Real-time PCR was the best performing single assay overall, particularly in regards to turnaround time and sensitivity, enabling clinical decisions to be made rapidly. In laboratories with adequate resources, it is a feasible alternative to using toxin and GDH EIAs for the detection of toxigenic *C. difficile*.

Not all laboratories have the required resources such as money, time and facilities to be able to perform specialised testing such as PCR. The increased cost of PCR over that of a toxin EIA (>\$20.00 vs. ~\$7.00) may make PCR an unlikely option for a routine diagnostic assay for CDI. It is possible that specimens could be sent to a reference laboratory for testing, but this too poses problems as turnaround times are affected.

Culture of *C. difficile* onto selective media following ethanol or heat shock of the stool would allow negative results to be issued within 48 hours, as culture is highly sensitive. In our study, ethanol shocking of the stool prior to plating on CDA selective agar improved the isolation rate compared with no pre-treatment of the stool and culture onto pre-reduced CDA selective agar (results not shown). Performing a highly sensitive and specific assay such as a PCR directly on the colonies to detect the toxin genes is a possibility and if performed the same day, would allow reporting of the positive cultures within 48 hours. The cost of culture is much less than PCR and toxin A/B EIAs and only culture positive samples would need further testing to determine whether the isolate is toxin producing.

Another possible option is to perform both a stool toxin A/B EIA and culture on all samples and interpreting the results of the two assays in combination. This combination would have detected all cases of CDI in our study. This option may be a feasible alternative and perform better overall than a multi-step algorithm. Some confirmatory testing may be required but this would be limited, particularly in a low prevalence setting. Delmée *et al* have

recommended simultaneously performing both culture and toxin A/B assay on stools (28). Samples with a positive culture but a negative toxin assay on stool would then have a toxin assay such as PCR performed on the isolate. If a combined testing protocol was used on the set of samples tested in our study, 100% of cases would have been detected and 97.7% of results could have been reported within 48 hours (100% specificity and sensitivity). Few specimens would require further testing such as PCR or EIA on the isolate, keeping costs reasonably low for the majority

Many practitioners start empirical treatment for *C. difficile* infection before any laboratory results are issued and, ultimately, the diagnosis of *C. difficile* infection by the physician takes into account relevant clinical data in combination with laboratory results. However, false negative laboratory results due to the poor sensitivity of commercially available *C. difficile* toxin A/B and GDH EIAs could possibly increase the risk of cross-infections in health care facilities if these results are interpreted in isolation. Cross-infections will subsequently increase the financial burden on the health care system.

Introducing either a highly sensitive RT-PCR such as the LightMix Kit used in our study or the two-assay combination of toxin EIA and culture would increase the number of diagnosed cases of CDI and may lead to improved clinical outcomes due to appropriate patient management. The workload and costs will inevitably increase in laboratories that have historically used toxin A/B EIAs for *C. difficile* infection diagnosis, but if more reliable results are being reported, cost savings will be made elsewhere in the health system.

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### Author information

Angela D Horridge, BMLS, Senior Scientist<sup>1</sup>  
Mary F. Nulsen, PhD, Associate Professor in Medical Microbiology<sup>2</sup>  
Rosemary Ikram, FRCPA, Clinical Microbiologist<sup>3</sup>

<sup>1</sup>Immunochemistry Department, Medlab South Ltd, Christchurch, New Zealand

<sup>2</sup>Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, New Zealand

<sup>3</sup>Medlab South Ltd, Christchurch, New Zealand

### Author contributions

Angela Horridge performed all the analytical work and data analysis and wrote the article. Mary Nulsen and Rosemary Ikram contributed to the planning and execution of the study and the substantial drafting of the article. The authors declare no conflicts of interest.

### Author for correspondence

Angela Horridge, Immunochemistry Department, Medlab South Ltd, Christchurch, 137 Kilmore Street, Christchurch, New Zealand. E-mail: angela\_horridge@medlabsouth.co.nz



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# A non-mosaic ring chromosome 9 in a newborn baby referred for ambiguous genitalia: a case study

Roberto L.P. Mazzaschi, Donald R. Love, Alice George and Salim Aftimos

## Abstract

A newborn child with ambiguous genitalia was referred for cytogenetic analysis. Routine karyotype analysis of cultured peripheral lymphocytes showed the presence of a ring chromosome 9 in 94% of cells. Karyotype analysis of the parents confirmed that this chromosome rearrangement was *de novo*, and a preliminary result was issued for genetic counseling purposes. A more detailed molecular karyotype was carried out, which showed a loss of 6.7Mb and 1.3Mb from the short and long arms of chromosome 9, respectively, resulting in an apparent single copy of approximately 70 genes. The infant died at 8 weeks of age from heart failure. Based on previously published cases, it is likely that both the heart defect and the ambiguous genitalia were caused by the loss of genes on the abnormal ring chromosome.

## Key words:

Cytogenetics, karyotype, ring chromosome, microarray, ambiguous genitalia

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## Introduction

The incidence of chromosome ring formation in humans is quite rare, with an estimated frequency of 1 in 50,000 human fetuses (1). The mechanism for this chromosomal abnormality remains unknown at present but it is strongly associated with the varying loss of the telomere ends on each arm of the chromosome. Critically, telomeres confer stability to chromosomes by preventing DNA degradation and end-to-end fusion during replication. Loss of this function results in unstable chromosomes which, in ring chromosome formation, result in annealing of the "sticky ends" thereby preventing further loss of DNA, but resulting in an unbalanced genome.

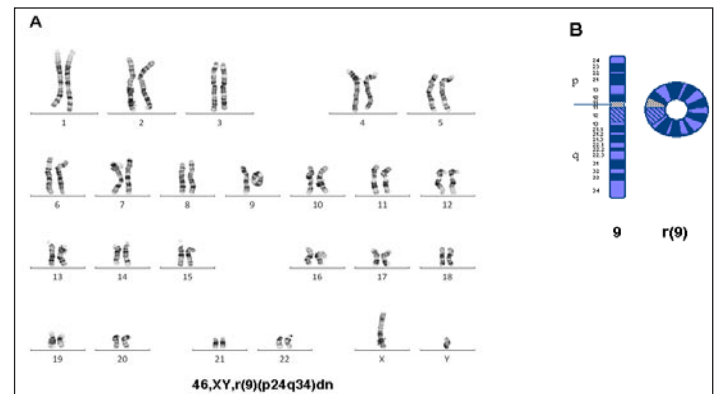
Normal human chromosomes are linear in structure consisting of two "arms" (designated "p" and "q") that flank a single active centromere. In addition to this, the ends of the chromosomes are "capped" with structures called telomeres, which give the chromosomes stability by preventing degradation of the ends during DNA replication. Rarely, ring chromosomes are formed, which occur at an estimated incidence of 1/50,000 in human fetuses (1). The mechanism of formation is unclear, but involves the loss of varying extents of the telomeric ends of the p and q arms, followed by annealing of the "sticky" ends in order to prevent further loss of genetic material. Although a ring chromosome is considered to have effectively regained its stability, the genome is unbalanced due to the loss of DNA. If this loss encompasses actively transcribed genes, then the resulting monosomy may lead to a phenotypic abnormality.

Ring chromosomes are divided into two groups: those in which one normal chromosomal homologue is replaced by a ring equivalent, and those in which the ring chromosome is additional to the two normal copies of that chromosome; such a ring is termed a supernumerary. Furthermore, many reported ring chromosome cases are mosaic in which the individual possesses at least two cell lines of varying chromosomal composition: typically a normal cell line without the ring chromosome, and another with a ring

chromosome. In addition, the proportion of these cell lines may vary between tissues, which further complicate the molecular confirmation of a clinical diagnosis and the prognosis.

Only 20 cases of ring chromosome 9 have been reported in the literature (2). Most of the breakpoints in each arm are not highly resolved, so genotype:phenotype classifications are difficult to make. However, some clinical features are common, including short stature, microcephaly, mental retardation and seizures. Less common features include heart defects, ambiguous male genitalia and cleft palate.

The case presented here involved an initial diagnosis of cleft palate, but with ambiguous genitalia; post mortem analysis at 8 weeks of age also identified a heart defect and renal cysts. Conventional and molecular karyotyping led to genetic counseling for the parents and high resolution identification of the extent of the genomic loss on the ring chromosome 9.



**Figure 1. Karyotype of the proband**

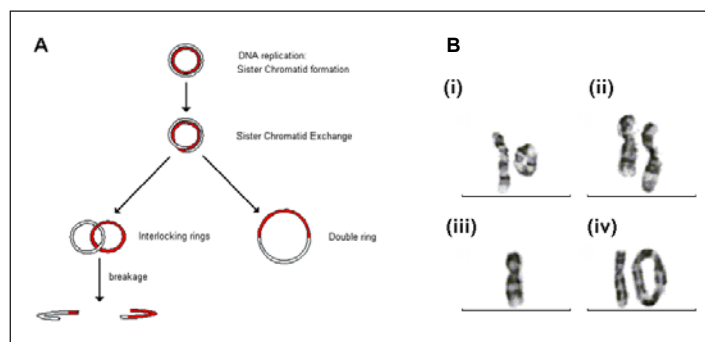
Panel A shows the 46,XY,r(9)(p24q34)dn karyotype of the proband. Panel B is a diagrammatic representation of the normal and ring chromosomes 9 in the proband.

## Case report

The infant was delivered at 38 weeks gestation after induction of labour because of clinical and sonographic detection of growth deceleration during the last few weeks of pregnancy. At birth, all growth parameters were below the third centile. The baby was admitted to NICU (Neonatal Intensive Care Unit) with hypoglycemia, and feeding difficulties. On examination, he was noted to have a posterior cleft palate, microphallus with hypospadias, with both testes high in the underdeveloped scrotum.

A request for cytogenetic analysis (see below) was based on the clinical presentation of ambiguous genitalia, cleft palate, pre and postnatal growth retardation. Antenatal ultrasound did not detect any major organ anomalies. However, at 8 weeks of age, the infant was admitted to hospital with respiratory distress and later died. The cause of death was cited as heart failure, secondary to patent ductus arteriosus. Subcapsular renal cysts were also noted sonographically at demise.

When the child was one day of age, we received a peripheral blood sample in lithium heparin for routine cytogenetic analysis (conventional karyotyping). Culturing was carried out according to standard protocols. The initial analysis of 30 cells showed a non-mosaic male karyotype with all cells carrying one normal homologue of chromosome 9 and one ring chromosome, 46,XY,r(9)(p24q34). Increasing this analysis to 70 blood lymphocyte metaphases identified a low level of mosaicism with two cells showing a loss of the ring 9 (resulting in monosomy for chromosome 9), one cell with a broken ring 9, and one cell with a double ring resulting in trisomy for chromosome 9 (Figure 2). Subsequent cytogenetic analysis of peripheral blood of the parents showed that they carried two normal copies of chromosome 9; hence the child's ring chromosome was *de novo*.



**Figure 2. Mosaicism of ring chromosome 9**

Panel A shows the outcomes of sister chromatid exchange (SCE) involving a ring chromosome. An even number of SCEs in the same direction can lead to normal symmetrical segregation of chromatids; an even number of SCEs in different directions can lead to interlocked rings, but only the breakage of the rings will allow them to segregate; an odd number of SCEs can lead to the loss of the ring in one cell, and two parallel chromatids forming a double-sized ring in the other. This panel is adapted from <http://atlasgeneticsoncology.org/Deep/RingChromosID20030.html>.

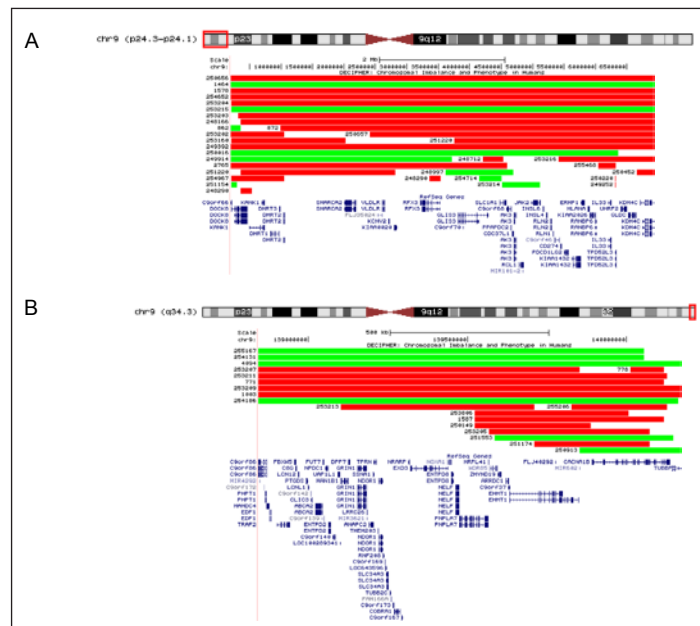
Panel B shows the normal and variant chromosomes 9 in some of the cultured cells of the proband; these cells comprised approximately 6% of 70 cells that were examined: panels (i) one normal chromosome 9 and one ring 9, (ii) one normal chromosome 9 and one broken ring 9, (iii) one normal chromosome 9 and a loss of the ring 9, and (iv) one normal chromosome 9 and one "double ring 9".

Based on the above karyotype data, the parents received genetic counseling. As neither of them carried the ring chromosome 9, it was considered extremely unlikely that the rearrangement would recur in future pregnancies. The couple already had three children, and as they were healthy, it was not considered necessary for them to be karyotyped. With regard to their baby, the parents were informed that previously reported cases with this very rare chromosome constitution presented with moderate to severe learning problems in later life, but with a variable degree of severity.

An EDTA blood sample from the child was requested for molecular karyotyping in order to determine the extent of the loss of chromosome 9 material and provide more informed counseling for the parents. DNA was extracted from the blood sample and genome-wide copy number analysis was determined using an Affymetrix® Cytogenetics Whole-Genome 2.7M array, according to the manufacturer's instructions. Regions of copy number change were calculated using the Affymetrix Chromosome Analysis Suite software (ChAS) v.1.0.1 and interpreted with the aid of the UCSC genome browser (<http://genome.ucsc.edu/>; Human Mar. 2006 (hg18) assembly).

The array analysis confirmed the initial cytogenetic findings and refined the breakpoints in both the "p" and "q" arms of the ring chromosome (Figure 3). The "p" arm had a 6.7Mb terminal deletion

encompassing the interval 199,112bp-6,926,078bp at chromosome regions 9p24.3 to 9p24.1. The "q" arm had a terminal deletion of 1.3Mb encompassing the interval 138,842,015bp-140,171,337bp at chromosome region 9q34.3 (Figure 3); it is likely that the deletions extended to the extreme telomeric end of both arms.



**Figure 3. Extent of the chromosome loss in the ring chromosome 9**

Panels A and B show the ideograms of chromosome 9 and the extent of genome loss on the p and q arms, respectively (taken from the UCSC genome browser <http://genome.ucsc.edu>). Deletions (shown in red) and duplications (shown in green) that are found at the terminal ends of chromosome 9 in other cases (reported in the DECIPHER database) are shown, together with the RefSeq genes that are localized to these regions.

## Discussion

Cytogenetic analysis is a standard test for a referral of ambiguous genitalia. In most cases where there is cytogenetic involvement, it usually involves the rearrangement or loss of a sex chromosome (X or Y). The case reported here represents a much rarer example of an autosome (non-sex chromosome) rearrangement interfering with normal sexual development. To aid genetic counseling, the first step involved a determination of any chromosomal rearrangements in the proband using conventional karyotyping, together with parental analysis to determine the inheritance characteristics of the rearranged chromosome. If one of the parents had a copy of the rearranged chromosome and was phenotypically normal, then it would be less likely that the ring chromosome would be the cause of the physical abnormalities found in the baby. Once it was established that this was a *de novo* rearrangement, it was important to confirm that it was the only cell line present in the baby. The presence of a normal cell line with no ring chromosome 9, for example, could improve the child's prognosis. The analysis of 70 metaphase cells ruled out significant mosaicism, at least in peripheral blood. A few anomalous cells were found, which might reflect a culturing artifact, or might be due to dynamic mosaicism (3). Dynamic mosaicism occurs as a consequence of sister chromatid exchange (SCE) of the ring chromosome during replication, resulting in several unbalanced chromosome outcomes. The continual production of these abnormal cell lines has the effect of growth retardation in carriers of ring chromosomes.

Subsequent microarray analysis linked our case to previously reported cases in the DECIPHER database (ref 4; <http://decipher.sanger.ac.uk/>). These other cases had similar deletions in either the p or q arms, and so were monosomic for only one end of chromosome 9, in contrast to our patient who was monosomic for both ends of chromosome 9. Our data, together with a literature

review of terminal deletions for chromosome 9 (Table 1), shows that sex reversal and ambiguous genitalia are associated with deletions of 9p, while deletions in both the p and q arms can lead to cardiac abnormalities.

### Conclusions

Routine cytogenetic analysis should be one of the initial tests carried out for referrals of ambiguous genitalia, as an underlying cytogenetic abnormality may be the cause. This case highlights the rare event of an autosomal rearrangement interfering with normal sexual differentiation. Molecular karyotype analysis offered a much higher level of resolution than traditional cytogenetic analysis. It provides more accurate breakpoint data and identifies the extent of deletion/monosomy of a ring chromosome. This in turn helps in the genetic counseling of a family, as more accurate predictions of phenotype can be made based on gene loss.

### Acknowledgments

We acknowledge the technical assistance of Shalinder Singh, Michel Qorri and Amel Al-Murrani for conventional and molecular karyotyping data.

### Author information

Roberto L.P. Mazzaschi BSc(Hons), Medical Laboratory Scientist<sup>1</sup>  
Donald R. Love PhD, FRCPath, FFSc(RCPA), Director<sup>1</sup>  
Alice George BSc(Hons) GradDipQS, Technical Head<sup>1</sup>  
Salim Aftimos MD, Pediatrician<sup>2</sup>

<sup>1</sup>Diagnostic Genetics, LabPlus and <sup>2</sup>Northern Regional Genetic Service, Auckland City Hospital, Auckland, New Zealand

### Author contributions

Roberto Mazzaschi contributed significantly to the analytical work and substantially drafted the article. Donald Love and Alice George substantively wrote parts of the article for critical content. Salim Aftimos conceived the study. The authors declare no conflicts of interest.

### Corresponding author

Alice George, Diagnostic Genetics, LabPlus, Auckland City Hospital, PO Box 110031, Auckland 1148, New Zealand. Email: AliceG@adhb.govt.nz

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# Simport

# Anatomical Pathology

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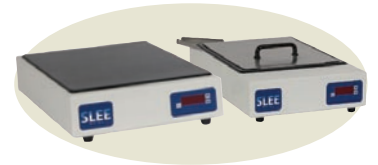
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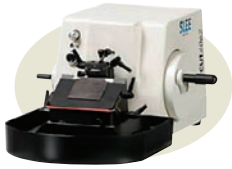
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# Letter to the Editor

## Prevalence of extended spectrum $\beta$ -lactamase among Gram-negative bacteria

### To the Editor

I am writing in regards to the article "Prevalence of extended spectrum  $\beta$ -lactamase among Gram-negative bacteria isolated from surgical wound and blood stream infections in Benin City, Nigeria" published in the November 2010 issue (1). I was disappointed at the quality of this article and felt that there were a number of serious shortcomings in the article that ought to have been addressed prior to publication. I am not a specialist microbiologist and therefore I have only noticed the errors apparent to someone not working in microbiology, there may be other errors besides those I have listed.

My first concern is that the text of the article contains information not supported by the data provided in the Tables. Under the results section the authors' state: "... the genera *Enterobacter*, *Proteus*, *Providencia* and *Alcaligenes* that produce ESBL were resistant to all the antibacterial agents used ...". The data in Tables 1 and 2 do not support this. In fact, according to Table 1 no ESBL producing *Alcaligenes* species were isolated. Table 2 shows that the two *Acinetobacter* species were resistant to all five agents and also that one of the two *Proteus* isolates was susceptible to amoxicillin-clavulanate, although as explained later in the article this may have been in-vitro activity only.

These discrepancies were highlighted by question 4 of the journal questionnaire, which asked, "Which genera that produce ESBL were resistant to all the antibacterial agents used in the study by Omoregie et al" and caused some concern for my colleagues.

My second concern is about the comparison of prevalence of different ESBL producing species in the discussion section. The authors state: "Although most studies have showed (sic) *Escherichia coli* and *Klebsiella* spp as the most prevalent ESBL producing Gram-negative bacteria, *Enterobacter* spp was the most prevalent ESBL producing Gram-negative bacteria from surgical wounds, while *Escherichia coli* and *Acinetobacter* were the most prevalent ESBL producing bacteria from blood stream infections in our study. The reasons for these differences are unclear."

This statement is not supported by the data in Table 1 which show *E. coli* was the most prevalent ESBL producing isolate in surgical wounds in the study (7 of 16 isolates, versus 2 isolates of *Enterobacter* spp.). With regards to the different species isolated from blood stream infections in the study, a data set of six isolates is insufficient to draw any meaningful conclusion on relative prevalence. In fact, the entire dataset of 22 ESBL producing isolates was very small and caution should be applied interpreting this data. However, it is interesting to note from the combined dataset that the two most prevalent ESBL producing isolates were *E. coli* and *Klebsiella*. That is to say, the data presented, although limited, is in line with the findings of previous studies, in contrast with the comments made by the study authors.

My final concern is the format of Table 1, where the headers for each column are clearly incorrect. For example, the column titled "No of ESBL producers" contained data for "Total number of surgical wound isolates", the column titles "Surgical wound" contained data for "ESBL producing surgical wound isolates." These misaligned column titles make interpreting the data difficult, and required the reader to work backwards from the data to identify what the actual column header should have been.

While the misalignment of column headers is perhaps an understandable typographical error, the discrepancy between the analysis in the text and the data in the tables is a serious problem, as is the attempt to draw conclusions on relative prevalence from a very small dataset. This was a poorly written article and while the overall conclusions about prevalence of ESBL Gram-negative bacteria may have some value, this was lost in the poor presentation and the clear discrepancies between the text and the data provided. As a result of these obvious errors, I have no confidence in the information presented in this article and find it particularly disappointing that, in spite of these shortcomings, it featured in the journal questionnaire. This article was not of the standard I would expect of the *New Zealand Journal of Medical Laboratory Science* and I urge more editorial care in the future.

### Reference

1. Omoregie R, Igbarumah IO, Egbe CA, Ogefere HO, Ogbogur PI. Prevalence of extended spectrum  $\beta$ -lactamase among Gram-negative bacteria isolated from surgical wound and blood stream infections in Benin City, Nigeria. *N Z J Med Lab Sci* 2010; 64: 74-6.

**Stewart Clark**  
Laboratory Manager  
Hutt Valley DHB

### The first author responds

Stewart Clark makes various comments on our recently published article in the Journal. In regard to his first comment we agree that we should have stated that the genera that produce ESBL should have mentioned *Acinetobacter* and not *Alcaligenes*, thus the correct sentence should read "While the genera *Enterobacter*, *Proteus*, *Providencia* and *Acinobacter* that produce ESBL were resistant to all the antibacterial agents used ...". We apologise for this oversight and thank Clark for pointing this out.

We do not agree with his comment about the comparison of prevalence of different ESBL producing species in the discussion section. A careful perusal of Table 1 indicates that *Enterobacter* species had the highest prevalence of 18.2% while *Escherichia coli* and *Klebsiella* species had prevalences of 5.6% and 2.6% respectively, from surgical wounds. Also, *E.coli* is 7 of 126 (not 16) and *Enterobacter* spp is 2 of 11. He also claims that the combined data set showed *E.coli* and *Klebsiella* spp as the most prevalent ESBL producers. Looking at Table 1, *E.coli*, *Klebsiella* spp and *Enterobacter* spp had total ESBL prevalence of 7%, 2.9% and 10.5% respectively and we stand by our comments.

While we would agree that the number of positive ESBL was small, statistical analysis with appropriate statistical test, such as odds ratios, are designed for small data from which meaningful deductions can be made. Therefore, we believe that our deductions are correct. We did comment in the Discussion section that this was a possible limitation of our study. We agree that the headers in Table 1 potentially pose a problem.

**Richard Omoregie, MPhil MSc FIMLS**  
School of Medical Laboratory Sciences, University of Benin  
Benin City, Nigeria

## **The Editor comments**

Stewart Clark has made various comments regarding the recent article by Omoregie *et al* in the Journal (1). The Journal welcomes Letters to the Editor regarding recently published articles. As per Journal policy, the authors were invited to respond and have done so. We leave it to the readers to determine whether the 1<sup>st</sup> author's response is based on scientific facts.

There were, however, various comments made by Stewart Clark that, as Editor, I feel compelled to respond to. He states that there were a number of serious shortcomings in the article that ought to have been addressed prior to publication and urged more editorial care in the future.

Every article submitted to the Journal undergoes peer review. Given the multidisciplinary nature of medical laboratory science, the Editor and Editorial Board Members cannot be expert in all disciplines and are reliant on peer review by experts in the article's subject. The article by Omoregie *et al* underwent peer review by two microbiology experts who made suggestions that the authors incorporated in their final article. Both advised acceptance of the article, subject to minor modifications. Additionally, the article underwent review by one of the Journal's Statistical Editors, who commented that the statistical analysis was appropriate for the study.

Thus, as Editor, I was satisfied that this article was of sufficient scientific merit to be published in the Journal.

**Rob Siebers, FNZIMLS FNZIC FSB**  
**Editor, New Zealand Journal of Medical Laboratory Science**

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# North Island Seminar 2011 – A night and day in the museum

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The North Island Seminar was held on the 28<sup>th</sup> of May in Wellington at Te Papa Museum. Wellington turned on a lovely sunny day for our guests from all over the country. Te Papa's Oceania Room was a great venue for the 196 delegates with plenty of space, delicious food and efficient service.

There was an interesting array of speakers covering all manner of laboratory and medical subjects. The standard of the presentations was excellent and many of these were first time speakers, giving the judges a difficult task.

Best overall speaker was awarded by Abbott to Jacqueline Case of Middlemore Hospital, Auckland for her presentation entitled "A trio of genetic abnormalities in one family". Best first time speaker was awarded by BioRad to Sarah Scoullar of Aotea Pathology, Wellington for her presentation entitled "A big fat paradox".

Drinks were served at Icon's for those keen to carry on catching up with colleagues, followed by a sumptuous BBQ dinner cooked out on the deck overlooking the harbour. It was a lovely evening and I think nearly everyone got up for a dance afterwards!

*Nicky Beamish - Convenor*

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## Presentations

### An understanding of the cell biology of infantile haemangioma leads to a new treatment and elucidation of the origin.

**Dr Darren Day**  
**Victoria University/Hutt Hospital**

Infantile haemangioma (IH), a primary tumour of the microvasculature, is the most common tumour of infancy, affecting about 10% of white children. Typically it undergoes postnatal growth during infancy (proliferating phase) characterized by excessive angiogenesis, followed by spontaneous involution over the next 1.5 years (involuting phase) with continued fibro-fatty deposition that replaces the cellular elements up to 10 years of age (involved phase).

The origin of IH is unclear, but it has been shown to express markers of both endothelial and haematopoietic lineages, and a role for endothelial progenitor cells in the aetiology of IH has been suggested. Evidence will be presented to demonstrate that IH is derived from extra-embryonic tissue that we hypothesize embolizes in the developing foetus. Our model is able to account for both the common focal lesions and those associated with

PHACES syndrome. Additionally, characterization of the stem cell population within IH has identified a key regulatory role for the renin-angiotensin system (RAS) in regulating IH progression, that has led the development of new therapeutic treatments for this disorder.

### Pathogen discovery **Dr Richard Hall – ESR Wallaceville**

For every vertebrate species it has been surmised that there may be 20 viruses that are unique to each of these species. The present number of classified viral species stands at approximately 2000, thus leaving the vast majority of viruses undiscovered.

Exciting new techniques, including MassTag PCR, microarray and next-generation sequencing have been trialled at ESR for the discovery of new or emerging pathogens (bacterial / viral / fungi / parasite), as well as for application to existing routine diagnostics. This work is being established at ESR in collaboration with the Center for Infection and Immunity (CII) at Columbia University, New York, which has recently been established as a new World Health Organisation Collaborating Center for Emerging Infectious and Zoonotic Diseases.

A staged discovery platform has been developed at the CII. Initial screening for common pathogens is achieved using MassTag PCR, which can detect 20 – 30 agents in a single test, such as for agents of viral hemorrhagic fever, bacterial enteric diseases or respiratory pathogens. If no infectious agent is detected then broad screening for all known vertebrate viruses can be applied using GreeneChip microarray or unbiased detection by next-generation (454FLX) sequencing. The use and application of these methods at ESR, in collaboration with Columbia University, will be presented.

### Influenza surveillance in NZ **Dr Sue Huang – ESR Wallaceville**

In 2009, pandemic influenza A(H1N1) 2009 (pH1N1) was imported into New Zealand which triggered the first pandemic in 41 years. This presentation will describe the influenza surveillance data based on ILI GP consultations, notifications, hospitalisations, mortality and virological surveillance. Decision on the influenza vaccine strain selection will also be described.

In addition, this presentation will describe a randomized cross-sectional seroprevalence study on pH1N1 in the general population and healthcare workers (HCWs) in New Zealand. This study was essential to understand population immunity, incidence of infection and risk factors of pH1N1. This would provide evidence for decisions on effective vaccination and other public health interventions as well as disease modelling. This presentation will describe the study methods and major findings. Overall, about 29.5% of New Zealanders had antibody titers at a level consistent with immunity to 2009 H1N1. Around 18.3% of New Zealanders were infected with the virus during the first wave including about one child in every three. Older people were protected due to pre-existing immunity. Age was the most important factor associated with infection followed by ethnicity. Healthcare workers did not appear to have an increased risk of infection compared with the general population.

### Can I catch cancer? **Dr Diane Kenwright – Capital Coast DHB**

Tumours, like microbiological organisms, are self replicating cells, programmed to survive at all costs. Unlike organisms they are rarely spread between individuals. There are specific circumstances however where tumours can be transmitted between normal



individuals. This presentation discusses the body mechanisms to defend against foreign tumour cells, instances where tumours have been transmitted to lab workers, experiments in transmitting tumours and why the Tasmanian Devil is in danger of extinction.

## **Infertility, hormones and IVF** **Winnie Duggan – Fertility Associates**

A typical infertility process and the many options for treatment in New Zealand. How hormone levels and monitoring of patients can impact on outcomes.

## **Shiftwork, stress and fatigue** **Ruth Beeston – Capital and Coast DHB**

Medical laboratory science requires many staff to participate in a shift roster to provide 24 hours service. What implications does this have for the health of the staff and the quality of the work? Whose responsibility is this and how can negative effects be mitigated?

## **A case of New Delhi metallobeta-lactamase** **Rachel Roth – Capital and Coast DHB**

A case presentation of NDM-1, and an overview of Carbapenemases

## **The big fat paradox** **Sarah Scoullar – Aotea Pathology**

The aim is to explain the importance and use of cholesterol in the body and present a case study where a patient has experienced a paradoxical decrease in their HDLc levels whilst on statins.

## **Specific IgE sensitisation in a six yr old infant cohort in NZ** **Assoc Prof Rob Siebers – University of Otago, Medical School, Wellington**

The aim of the study was to determine sensitisation to inhalant and food allergens in 6-year old children in New Zealand. Specific IgE (sIgE) levels were determined to 12 inhalant and food allergens in 659 6-year old children from Wellington (n=316) and Christchurch (n=343) from a birth cohort. Allergens tested were *D. pteronyssinus*, cat pelt, dog hair, horse hair, cockroach, *A. fumigatus*, *Alternaria*, olive pollen, rye grass, egg white, cow's milk and peanut by a 3rd generation liquid chemiluminescent enzyme immunoassay (Siemens IMMULITE 2000). Atopic sensitisation was defined as at least one sIgE  $\geq$  0.35 kU/L. Results are presented as proportions and geometric means with 95% confidence intervals (95% CI). Sensitisation to inhalant and food allergens is high in New Zealand 6-year old children with sensitisation to *D. pteronyssinus* the most prevalent and with very high IgE levels and specific IgE activity.

## **A trio of genetic abnormalities in one family**

### **Jacque Case – Middlemore Hospital**

An acute admission of a young girl with symptoms of a haemolytic process revealed more surprises!

## **I've got you under my skin (and other places)**

### **Brian Allred – PathLab Waikato**

A brief presentation of 3 parasitology case studies

## **What can go wrong with your research?**

### **Assoc Prof Mike Legge – University of Otago, Dunedin**

When planning a research project, the idea is usually really good but part way down the track not everything comes together! In this presentation thoughts on planning the research project and where things can go wrong (both innocently and deliberately) will be considered. Some examples of deliberative scientific deception in published research will be considered in the context of this presentation.

## **RTA and its effect on error rates in lab sample registration**

### **Dave Currie - Aotea Pathology**

In 2009 Aotea Pathology introduced a real time audit procedure to ensure that all visits entering the laboratory had their important details checked before being released to the testing department. This talk will examine the error rates before and after real time audit implementation, the process of introducing this new system, and the effect on the laboratory as a whole.

## **The day I died - review of CAD and case of acute MI episode**

### **Tony Mace - Pathlab Waikato**

This is a review of coronary artery disease (CAD) and a case study of an acute MI episode.

## **New test, new analyser, inhibin A, Beckman Access**

### **Melvina Wise - Lab Plus Auckland**

Clinical chemistry recently introduced the test inhibin A. This test has become part of the second trimester maternal serum screening program done through National Testing Centre. This required the introduction of a new analyser, the Beckman Access. This presentation will take you through the problem solving process involved to get Inhibin A up and running.

## Muscular dystrophy - role of muscle stains

### Durendra Sami – LabPlus Auckland

Muscular dystrophy (MD) is a genetic muscular disorder characterised by progressive weakness and degeneration of the skeletal muscles that control movement. It is caused by absence of a gene or a mutated gene which is responsible for producing dystrophy. Clinically patients present with weakness and wasting of muscle and a lack of ability to walk. There are nine major types of muscular dystrophies. Duchenne muscular dystrophy (DMD) is the most common type.

Cryostat cut sections stained with haematoxylin/eosin, histochemical stained sections and dystrophin immunohistochemistry play an important role in establishing a definite diagnosis of various subtypes of muscular dystrophy. The aim of this presentation is to show a comparison of normal muscle biopsy sections and muscular dystrophy sections. This presentation will aim at raising issues affecting testing.

## Journal Questionnaire

Below are 10 questions based on articles in the August 2011 Journal issue. Read the articles fully and carefully, most questions require more than one answer.

Answers are to be submitted through the NZIMLS web site. Make sure you supply your correct email address and membership number. It is recommended that you write your answers in a word document and then cut and paste your answers on the web site.

The site has been developed for use with Microsoft's Internet Explorer web browser. If you are having problems submitting your questionnaire and you are using the Firefox web browser, try resubmitting from a computer or system using Microsoft's Internet Explorer.

You are reminded that to claim valid CPD points for successfully completing the Journal questionnaire you must submit an individual entry. It must not be part of a consultative or group process. In addition, members who have successfully completed the Journal questionnaire can only claim 5 CPD points. You can not then claim additional CPD points for reading the articles from which the questions were derived.

The site will remain open until Friday 4<sup>th</sup> November 2011. You must get a minimum of 8 questions right to obtain 5 CPD points.

### August 2011 journal questions

1. What is used as a marker of ESBL production and why.
2. Which antibiotics are ESBLs able to hydrolyse and which are they not able to hydrolyse.
3. What is involved in the mechanism of formation of ring chromosomes.
4. Name the two rings into which ring chromosomes are divided.
5. Dynamic mosaicism occurs as a consequence of what and what does it result in.
6. What should be one of the initial tests carried out for referrals of ambiguous genitalia and why.

7. Name the clinical symptoms associated with *C. difficile* infection.
8. What laboratory tests are available for the diagnosis of *C. difficile* infection.
9. Incorrect laboratory results for *C. difficile* infection diagnosis due to either lack of sensitivity or specificity can lead to what.
10. What was a possible limitation of the *C. difficile* study.

### Questions and answers for the April 2011 journal questionnaire

1. The resistance mechanism in bacteria to  $\beta$ -lactams is. **The production of  $\beta$ -lactamases that catalyze the hydrolysis of the  $\beta$ -lactam ring.**
2. What test was used for the phenotypic detection of AmpC producers. **Modified three-dimensional extract test.**
3. What are the only antibiotics effective against AmpC producing strains. **Carbapenems.**
4. Failure in the laboratory to detect AmpC producing strains may lead to what. **Inappropriate and unsuccessful therapy of the patient and unnecessary usage of drugs.**
5. What has also been reported as one of the resistance mechanism of cefoxitin in AmpC non-producers. **Lack of permeation of porins.**
6. Why is early identification of cefoxitin-resistant organisms necessary. **As the appropriate treatment might reduce the spread of these resistant strains and reduce mortality in hospitalized patients.**
7. Why should a genotypic test be used for detection of AmpC-producers. **Because the appropriate treatment might reduce the spread of resistant strains and consequently mortality in hospitalised patients can be reduced.**
8. What are the primary criteria in establishing and maintaining cell cultures. **Growth, morphology and response to experimental manipulation.**
9. Name two factors that can *in-vitro* induce modification of cells and what are their effects. **Fetal bovine serum and choice of buffer. Modification of cell glucose content and glycosylation.**
10. What is the main conclusion from the study by Hazlett and Legge. **That the concentration of fetal bovine serum has the potential to modify protein synthesis of at least five proteins *in-vitro*.**



# New Zealand Institute of Medical Laboratory Science

## The Barrie Edwards & Rod Kennedy Scholarships

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The Barrie Edwards & Rod Kennedy scholarships are some of the most significant awards offered by the NZIMLS. The two scholarships provide winners with support to attend an international or national scientific meeting up to a maximum value of \$7,500 for each.

Applications for these prestigious scholarships are invited from Fellows, Members and Associate Members of the NZIMLS. Applicants must be a current financial member of the NZIMLS and have been a financial member for at least two concurrent years prior to application. To be eligible applicants must make an oral presentation or present a poster as 1st author at their nominated scientific meeting.

All applications will be considered by a panel consisting of the President and Vice-President of the NZIMLS and the Editor of the New Zealand Journal of Medical Laboratory Science (who are ineligible to apply for the scholarships). The applications will be judged on your professional and academic abilities together with your participation in the profession. The panel's decision is final and no correspondence will be entered into.

Application is by letter. Please address all correspondence to:

**NZIMLS Executive Officer**  
**PO Box 505**  
**Rangiora 7440**

There may be two scholarships awarded in each calendar year. Closing dates are - June 30th and December 20th in any given year.

In your application letter please provide the following details:

- Full name, position, work address, email address and contact phone number
- The length of time you have been a financial member of the NZIMLS
- The conference you wish to attend - please provide dates

- A budget comprising airfares, conference registration and accommodation costs
- The abstract of your intended oral or poster presentation and whether it has been accepted for presentation (proof required)
- Your intentions to publish your results
- State briefly your history of participation in the profession over the last 5 years
- State the reasons why you wish to attend your nominated scientific meeting

Successful applicants will be required to provide a full written report on return which will be published in the Journal. If not intended to publish elsewhere, successful applicants will be required to submit their study results for consideration by the New Zealand Journal of Medical Laboratory Science.

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## Global Science Journal Award

Global Science & Technology Ltd. offers an award for the best article published during the calendar year in the *New Zealand Journal of Medical Laboratory Science* worth \$300. All financial members of the NZIMLS are eligible. The article can be an Original, Review or Technical Article. Excluded are Editorials, Reports, Fellowship Treatises or Case Studies (Case Studies are judged under the NZIMLS Journal Prize)

No formal application is necessary but you must be a financial member of the NZIMLS to be eligible. The Editor and Deputy Editor will decide in December which article is deemed worthy of the award. Their decision will be final and no correspondence will be entered into.

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# NZIMLS Journal Prize

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Council of the NZIMLS has approved an annual Journal prize (\$300) for the best case study published in the Journal during the calendar year.

Case studies bring together laboratory results with the patient's medical condition and are very educational. Many such studies are presented at the Annual Scientific Meeting, SIG meetings, and the North and South Island Seminars, yet are rarely submitted to the Journal for wider dissemination to the profession. Consider submitting your case study presentation to the Journal. If accepted, you are in consideration for the NZIMLS Journal Prize and will also earn

you CPD points. Please contact the Editor or any Editorial Board Member for advice and help. Contact details are on the NZIMLS web site ([www.nzimls.org.nz](http://www.nzimls.org.nz)) as are instructions to authors. Refer to the article "How to write a laboratory-based case study for the journal" published in the April 2010 issue of the journal, pages 22-23, for guidance.

No formal application is necessary but you must be a financial member of the NZIMLS during the calendar year to be eligible. All case studies accepted and published during the calendar year (April, August and November issues) will be considered. The Editor, Deputy Editor and the President of the NZIMLS will judge all eligible articles in December each calendar year. Their decision will be final and no correspondence will be entered into.

**Winner of the NZIMLS Journal prize for 2010 was Vichiet Khieng for his case study "Vitamin D toxicity? A case study". *N Z J Med Lab Sci* 2010; 64: 44-50.**

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# The Olympus Journal Imaging Competition

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**OLYMPUS**<sup>®</sup>

The NZIMLS Journal invites applications for the annual Olympus photo competition giving NZIMLS members the chance to win an Olympus digital camera and have their photo published in the journal.

The general theme is "Medical Laboratory Science", so whether it is related to haematology / histology, laboratory personnel, instruments, humour, or other, there's plenty of scope for

keen photographers to showcase their talents.

Olympus, a leading manufacturer of professional opto-digital products, has generously donated a digital camera as the prize for the best photo.

Entries should be submitted as an email attachment to Rob Siebers, Editor of the NZIMLS Journal, at [rob.siebers@otago.ac.nz](mailto:rob.siebers@otago.ac.nz). A title for the photo, together with the entrant's name, place of work and email address, should accompany the attachment. Submissions can be in colour or black and white.

Entries close on **5pm on Friday 16th September 2011**, with the winning photo appearing in the November 2011 issue of the Journal.

Judging will be carried out by the Editor, Deputy Editor and an Olympus representative. Their decision will be final and no correspondence will be entered into. Entrants must be current financial members of the NZIMLS to be eligible.

For further information about the competition, go to: [www.nzimls.org.nz](http://www.nzimls.org.nz)

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# Fellowship of the New Zealand Institute of Medical Laboratory Science

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The NZIMLS encourages members to consider Fellowship as an option for advancing their knowledge and career prospects. Fellowship provides an attractive option to academic postgraduate degrees at a fraction of the cost.



Fellowship of the NZIMLS may be gained by examination, by thesis or by peer-reviewed publications.

## Examination

Consists of two parts:

- α) Part 1: Two written papers each of three hours duration
- β) Part 2: Upon successful completion of Part 1 a dissertation of 3000 - 5000 words

The dissertation may take the form of a review, development of a hypothesis or any other presentation that meets with the approval of the Fellowship Committee.

## Thesis

The thesis must be based on the style of Master of Science by Thesis requirements of New Zealand Universities and not exceed 20,000 words.

## Publications

A minimum of seven peer-reviewed publications, of which the candidate must be first author of at least four, may be submitted for consideration. These need to have been published in international or discipline acknowledged scientific journals. A review of the submitted articles of 3000 – 5000 words must also be submitted. The candidate must state the contributions he or she made to the publications.

## Exemption

Candidates who are holders of postgraduate or professional qualifications in Medical Laboratory Science may be exempt from the Part 1 examinations but are still required to submit a dissertation for Fellowship.

Qualifications recognised by the NZIMLS for the purpose of exemption to sit the Part 1 examinations are:

- Fellowship of the Australian Institute of Medical Scientists (FAIMS), the Institute of Biomedical Science (FIBMS) and the Australasian Association of Clinical Biochemists (FAACB)
- An academic postgraduate qualification, normally at least a postgraduate diploma, in medical laboratory science or closely related subject. The course of study must meet the minimum requirement of one year's full-time study

For full Fellowship regulations and application process visit the NZIMLS web site: [www.nzimls.org.nz](http://www.nzimls.org.nz) or contact the Fellowship Committee Chair: Assoc Prof Rob Siebers at [rob.siebers@otago.ac.nz](mailto:rob.siebers@otago.ac.nz)



Dr M Shahid has been awarded Fellowship of the NZIMLS following submission of peer-reviewed publications and successful examination of his treatise "β-lactams vs. β-lactamases – recent evolution of concurrent occurrence of multiple *bla* genes including *bla*<sub>CTX-M</sub> and *bla*<sub>ampC</sub>: an Indian scenario" which is published in this issue of the Journal.

Dr. M. Shahid, MBBS, MD, PhD., FZNIMLS is currently working as Associate Professor in the Department of Microbiology of the J.N. Medical College & Hospital, Aligarh Muslim University, India. His field of interest and research is confined to the mechanism and resistance to beta-lactam antibiotics, with special interest in CTX-M and AmpC β-lactamases. He also has a research experience on human fungal pathogens such as *Aspergillus* and *Candida* species. His work on Aspergillosis in patients suffering from bronchogenic carcinoma has received high acclaim and recognition in the international scientific community. Moreover, his group is working in the field of histamine-research and produced some pioneer researches related to H1–H4 receptors and their agonists/antagonists. Dr. Shahid has published more than 75 research papers in reputed journals and has five books and more than 25 book chapters to his credit. He is presently the member of many scientific bodies, both international and national. He has also been the member of the reviewer panel and editorial board of various international journals/publication houses, including Lancet and Lancet Infectious Diseases; and also those of Bentham Science Publications, USA, and Global Science Books, UK. He has also worked as an Associate Professor in the College of Medicine, King Faisal University, Al-Hassa, Kingdom of Saudi Arabia; Section of Immunity & Infection, The Medical School, University of Birmingham; and NHS Heartlands Hospital, Birmingham, United Kingdom.

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### Greetings from the PPTC

This year has once again got off to a busy start. Here are some of the highlights of the first six months of 2011.

### Haematology and blood cell morphology

Another very successful haematology course was conducted in March with nine students from many of the Pacific Island countries. As in the past, the course provided students with guidelines for the microscopic evaluation of white cells, red cells and platelets in both health and disease. The students were introduced to the workings of the microscope in terms of correct operation and essential maintenance. They learnt the principles of Romanowsky staining, the preparation of stains and buffers, causes of inconsistent staining quality, the staining techniques used in the identification of malarial parasites, specimen quality, the effects of anticoagulants.



Students, staff and friends at the certificate presentation for the haematology course

### Diploma in Medical Laboratory Technology

Teaching of laboratory technology, the first module of the revised diploma, commenced in April through WHO's POLHN website. This module covers much of the basics of medical laboratory technology including the basics of organic chemistry and cell structure, laboratory instrumentation its uses and routine maintenance and laboratory safety. Thirty laboratory staff from Pacific Island laboratories have registered for this course.

### Haematology training in Tonga

In early May Phil Wakem, the Programme Coordinator travelled to Tonga for two weeks to conduct in-country training in haematology. The daily training sessions were attended by the majority of laboratory staff of the Vaiola Hospital in Nuku'alofa and so all were able to benefit and increase their skills. It is hoped that the frequency of this form of training will increase in the future. Phil returns to Tonga mid-June for a follow-up week.

### Strategic plan

The current 2007-11 strategic plan for the PPTC is about to come to an end and so in April the PPTC Board and staff spent some time with facilitators working on the next five year plan. Under the new strategy, the primary objective of the PPTC: "that its teaching and capacity building programmes must be appropriate, affordable and sustainable for the health care setting in which they will be used" will remain.

However, in addition to our current training programmes both here in Wellington and through distance learning we are looking at conducting more in-country training and also having a significant involvement with WHO in the implementation of their strategy for the strengthening of laboratory services in the Pacific region.

### PIHOA laboratory network meeting

In late May John attended this meeting in Guam. This network is made-up of all the laboratories in the US Associated Pacific Islands [USAPI]: Marshall Islands, Federated States of Micronesia, Palau, American Samoa, Guam and CNMI. This meeting is held every two years and this year's theme was "Laboratory Managers – Creating Solutions to Today's Problems." This was a very successful meeting and congratulations to Vasiti Uluviti, the PIHOA laboratory specialist, for great organisation. At the conclusion of this meeting the 2<sup>nd</sup> meeting of the Association of USAPI Laboratories [AUL] was held and many issues faced by these laboratories, including training and standards, were discussed.

### Fiji National University

We have been notified by FNU (previously the Fiji School of Medicine, School of Medicine, Nursing and Health Sciences that students who have completed their PPTC DipMLT through POLHN will be given credits for this and if they enter the BMLSc course at FNU will be exempted from some first year courses. We have been discussing this with FNU for some time and it is great news that this accreditation has now come through.

### PPTC AGM

This was held late May and during the meeting the Annual Report and Financial Report were presented and accepted. For the past 30 years Dr Ron Mackenzie has been chairman of the PPTC but this year he has resigned from this position although he still remains on the Board of Management. At the AGM Marilyn Eales made a presentation to Ron on behalf of the board, staff and friends of the PPTC and thanked Ron for the years of hard work that he had put into the founding of the Centre and then all the work he has done since in ensuring that it lived up to and met the objectives that had been set in the early days. The PPTC is what it is today because of the vision and hard work of Ron. This was acknowledged with applause by all those at the AGM. Mr Mike Lynch was elected as Chairman and the other Board members elected were Marilyn Eales, Dr Ron Mackenzie and Assoc Prof Rob Siebers.



Current members of the PPTC Board of Management  
From left to right: Assoc Prof Rob Siebers, Mike Lynch, Marilyn Eales, Dr Ron Mackenzie

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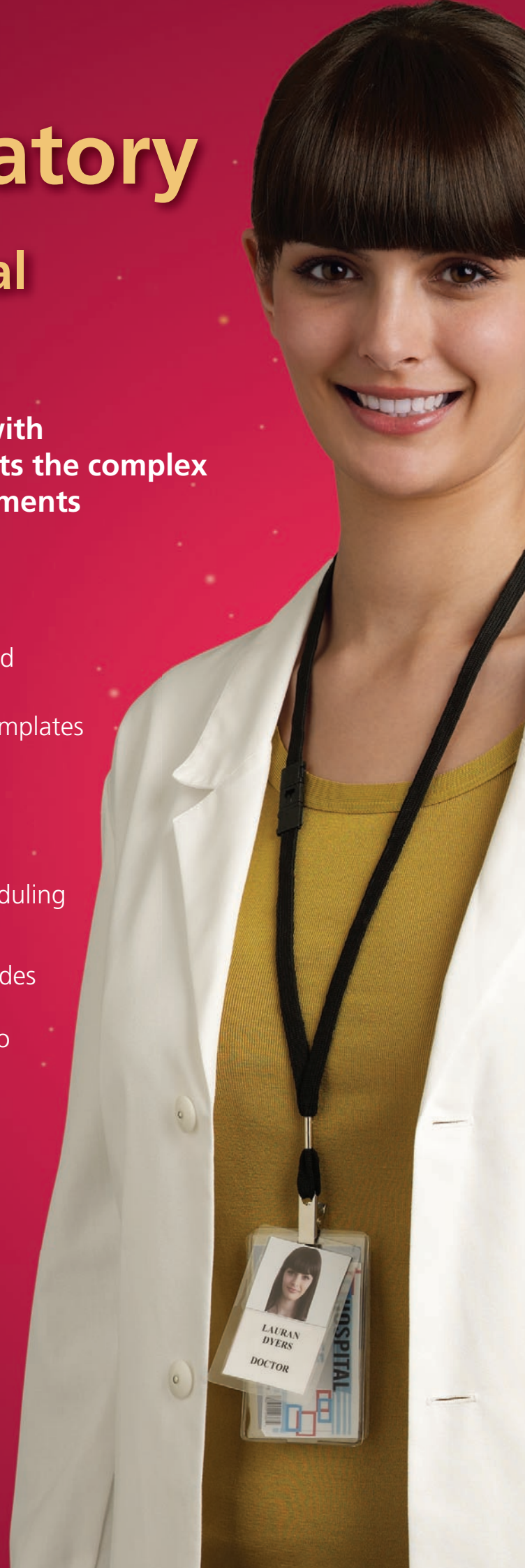
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